

**Glutathione-dependent-Enzyme Expression
In Drug Resistance**

Alexander David Lewis

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University of Edinburgh

1988



It is a melancholy of mine
own, compounded of many
simples, extracted from
many objects, and indeed
the sundry contemplation
of my travels, which, by
often rumination, wraps
me in a most humorous
sadness.

AS YOU LIKE IT.

Declaration

I hereby declare that:-

- a) this thesis has been composed by myself, and**
- b) the work contained herein is my own, unless
otherwise indicated.**

Abstracts and Presentations arising from Research

Lewis, A.D. and Wolf, C.R.

Changes in glutathione and glutathione-dependent enzymes during cell division.

Presented to the Scottish and Newcastle Drug Metabolism Group,
November 1985.

Lewis, A.D. and Wolf, C.R.

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Lewis, A.D., Hayes, J.D. and Wolf, C.R.

Expression of glutathione S-transferases in tumour cell lines.

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Lewis, A.D., Hayes, J.D., Hickson, I.R., Robson, C.N. and Wolf, C.R.

Expression of glutathione S-transferases in acquired drug resistant tumour cell lines.

Proc. American Association of Cancer Research, **28**: 283, 1987.

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The role of glutathione S-transferases in the resistance of cells to cytotoxic drugs.

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Lewis, A.D., Hickson, I.D., Hall, A., Robson, C.N., Harris, A.L., Hayes, J.D. and Wolf, C.R.

Elevated expression of an alpha class Yc containing glutathione S-transferase and γ -glutamyl transpeptidase in a CHO cell line resistant to bifunctional nitrogen mustards.

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Induction and suppression of glutathione S-transferases by interferon in the mouse.

J. Biol. Chem. **262**: 4888-4892, 1987.

Robson, C.N., Lewis, A.D., Wolf, C.R., Hayes, J.D., Hall, A., Proctor, S.J., Harris, A.L. and Hickson, I.D.

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The role of glutathione in determining the response of normal and tumour cells to anticancer drugs.

International Symposium on Glutathione and Cancer Therapy. Biochem. Soc. Transactions, **15**: 728-730, 1987.

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Wolf, C.R., Hayward, I.P., Lawrie, S.S., Buckton, K., McIntyre, M.A., Adams, D.J., Lewis, A.D., Scott, A.R.R. and Smyth, J.F.

Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient.
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I dedicate this thesis to the fond memory of my grandmother, the late Elizabeth S. Watson.

Abbreviations

GSH	:	reduced glutathione
GSSG	:	oxidised glutathione
γ GT	:	γ glutamyltranspeptidase
γ GCS	:	γ glutamylcysteinylsynthetase
GST	:	glutathione S-transferase
GPD	:	glutathione reductase
GPX	:	glutathione peroxidase
CDNB	:	1,chloro-2,4-dinitrobenzene
MDR	:	multidrug resistant (resistance)
BSO	:	buthionine S-R sulfoximine
Adr	:	adriamycin
MTX	:	methotrexate
CP	:	cyclophosamide
CHO	:	Chinese hamster ovary
SDS	:	sodium dodecyl sulphate
FACS	:	fluorescent activated cell sorter
4-OH-CP	:	4-hydroxycyclophosamide
EA	:	ethacrynic acid
H ₂ O ₂	:	hydrogen peroxide
CHP	:	cumene hydroperoxide
AFB ₁	:	aflatoxin B ₁
MESNA	:	mercaptoethane sulphonate
PBS	:	phosphate buffered saline
MBBr	:	monobromobimane
hplc	:	high performance liquid chromatography
MBq	:	megabequerels
MEM	:	minimum essential medium
DMEM	:	dulbecco modified eagle medium

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate Alexander David Lewis

Address ICRF, Laboratory of Molecular Pharmacology & Drug Metabolism, George Square, Edin.

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Title of Thesis Glutathione-Dependent Enzyme Expression in Drug Resistance

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No. of words in the main text of Thesis 40,000

Glutathione and glutathione-dependent enzymes play a central role in the protection of cells from cytotoxic chemicals. In particular, reduced glutathione (GSH) and glutathione S-transferases (GST's) have been studied in relation to intrinsic and acquired resistance of tumours to cytotoxic drugs. There are however, other glutathione-dependent enzymes which may also be involved in drug resistance: these include glutathione peroxidase (GPX) and the enzymes, γ glutamyltranspeptidase (γ GT), γ glutamyl cysteinyl synthetase (γ GCS) and glutathione reductase (GRD). The latter enzymes are involved in the maintenance of reduced GSH levels within cells. GSH and the above glutathione-dependent enzymes have been studied in a series of drug resistance models, a) drug resistant tumour cell lines generated *in vitro* and *in vivo*; b) chemotherapeutic agent induced resistance in normal bone marrow cells; c) in oxygen resistant lung cells and d) in preneoplastic foci, in order to evaluate whether GSH and associated glutathione-dependent enzymes form part of an adaptive response involved in protection against the environment.

Several models of drug resistance involving cell lines in culture were taken for study. A CHO cell line resistant to chlorambucil, an ovarian cell line generated *in vivo*, resistant to cis-Platinum and chlorambucil, and a sarcoma cell line resistant to adriamycin. In all these cell lines GST, GSH and γ GT were significantly elevated. In the latter two cell lines selenium dependent GPX was also induced. In the CHO line the elevated GST activity was explained by a 40 fold induction of the alpha class Yc GST subunit and a 2 fold elevation in the Ya subunit. In mouse bone marrow cells following the administration *in vivo* of a low 'primary dose' of cyclophosphamide, transient increases in alpha Ya and particularly mu Yb GST subunits were found. These increases have been associated with a subsequent protection against a higher lethal dose of the same agent. The changes observed in GST isozyme composition were confined to the granulocyte population.

Differences in selenium-dependent GPX, GSH and γ GT were also found in cells resistant to high oxygen, with only marginal changes in GST subunit profiles.

In preneoplastic foci, significant elevations in pi class Yf GST and also alpha Ya and mu Yb were detected. Selenium-dependent GPX was decreased and, γ GCS and GRD elevated in this model. These studies indicate therefore:

- 1) GST levels in certain cells appear to be directly related to resistance to cytotoxic chemicals;
- 2) Changes in GST expression associated with preneoplasia and in acquired drug resistance are not confined to one sub group of GST enzymes;
- 3) Changes in GST expression are often paralleled by changes in GSH and other glutathione-dependent enzymes;
- 4) The most consistent phenotypic changes observed in the drug resistant models studied were in GSH and γ GT levels;
- 5) There seemed to be an inverse relationship in the regulation of selenium and non-selenium-dependent GPX activity in different drug resistant models.

GSH and glutathione-dependent enzyme changes in acquired drug resistance, appear to be due to an adaptive response which may be of central importance in the resistance of tumours to chemotherapeutic agents.

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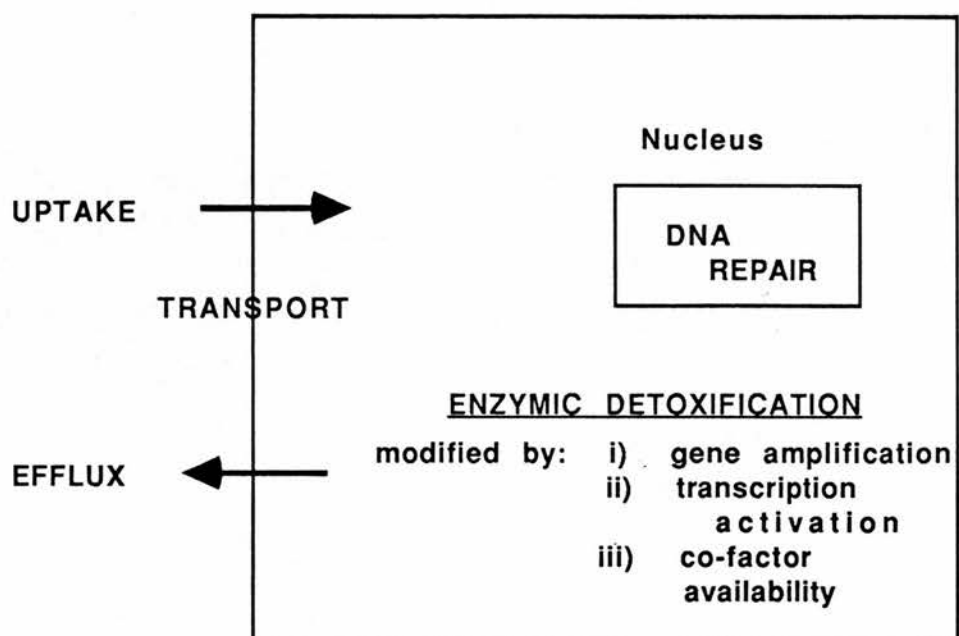
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A major problem in current cancer chemotherapy results from the ability of a small proportion of malignant cells to survive exposure to cytotoxic drugs. This type of drug resistance is categorised in several ways. In one situation, a neoplastic cell within the population becomes resistant to chemotherapy (intrinsic drug resistance). Alternatively, the cells may only become resistant after exposure to the cancer chemotherapeutic agents. This is generally known as acquired drug resistance. A wide variety of tumours fall into this latter category, for example, childhood leukaemia, breast cancer, and small cell lung cancer. The various mechanisms for these resistance phenomena have been postulated, as illustrated in Figure 1. These include decreased drug uptake by changes in drug-specific transport mechanisms (Kartner *et al*, 1983), decreased activation of prodrugs, alterations in the drug's target enzymes, alterations in cellular metabolism and repair mechanisms (Karran & Lindahl, 1985), and increased drug detoxification (Hayes & Wolf, 1988).

Glutathione (GSH) and glutathione-dependent enzymes play a central role not only in drug detoxification, but also in the protection of cells from toxic oxygen metabolites (Chasseaud, 1979; Larsson *et al*, 1983a; Wolf *et al*, 1987a, 1987c; Hayes & Wolf, 1988). These enzymes may, therefore, appear to be part of an early protection mechanism that evolved to accommodate respiration. The presence of GSH and glutathione-requiring enzymes in primitive organisms, such as bacteria and yeast, substantiate their very early development. GSH, however, is not essential for life, as certain bacterial mutants have been described lacking this tripeptide (Fuchs *et al*, 1983).

There are some recent reports indicating that GSH and glutathione-dependent enzymes may also play a role in drug resistance (Wolf *et al*, 1987a, 1987c; Hayes & Wolf, 1988). These studies have concentrated on reduced GSH and the glutathione S-transferases (GST's). However, many other glutathione-

Figure 1. Cellular Protection Mechanism



dependent enzymes may also be important in this phenomenon. These include proteins such as selenium-dependent glutathione peroxidase (GPX), γ glutamyl transpeptidase (γ GT), γ -glutamylcysteinylsynthetase (γ GCS), and glutathione reductase, all of which will determine the thiol status of the cell, ie. GSH:GSSG ratio, but some are also involved in detoxification. Thiol status is important, as many proteins which contain thiols at the active site - these being essential for cellular function - require reduced conditions for their active state (Harrap et al, 1973; Kosower et al, 1978). Whether differences in the levels of the above enzymes are a factor in drug resistance, is at present unclear. The respective roles of these proteins in cellular function, and their potential role in carcinogenesis and drug resistance, is discussed.

1.1.0 **Glutathione**

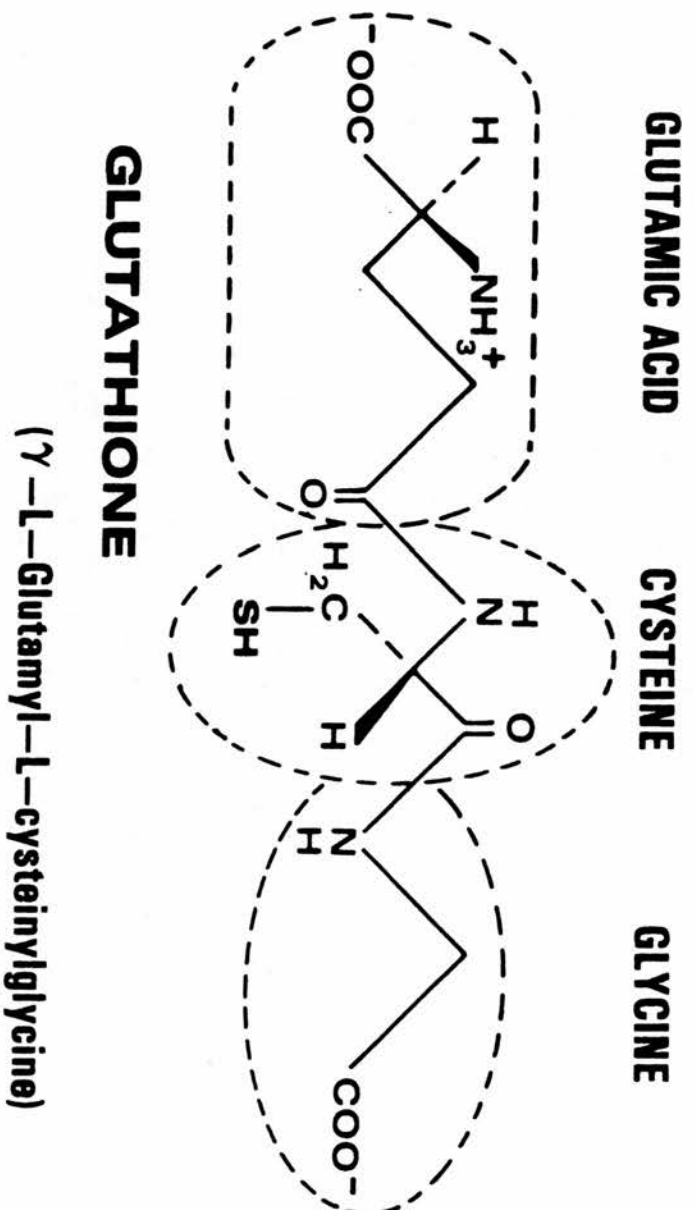
1.1.1 Historical

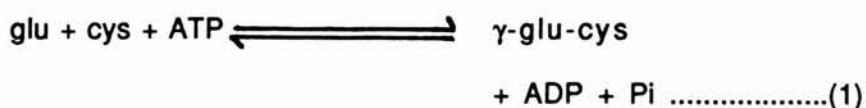
In 1888, de Rey-Pailhade (de Rey-Pailhade 1888a, 1888b) isolated a substance containing sulphur from yeast which he called "philothion". Some time later, Hopkins (1921) crystallised this compound, again from yeast, which he renamed glutathione. The chemical composition of glutathione was described by Pirie & Pinhey (1929) in 1929. Using different analytical techniques, Hopkins (1929) and Kendall et al (1929) also described this structure as γ -L-glutamyl-L-cysteinyl glycine (Figure 2). Glutathione was finally synthesised in 1935 by Harrington & Mead (1935).

1.1.2 Biochemistry of Glutathione (GSH)

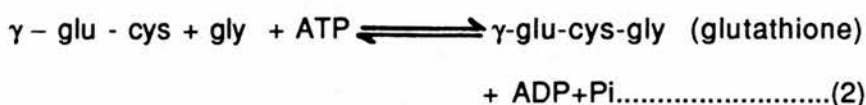
GSH is ubiquitous and is the predominant intracellular, non protein sulphydryl (NPSH), in both prokaryotic and eukaryotic cells (Larsson et al, 1983a). The biosynthesis of reduced GSH occurs in two successive ATP requiring steps (Meister, 1974). First, γ -glutamyl-cysteinyl-synthetase catalyses the formation of an amide linkage between cysteine and the γ -carboxyl of glutamate (equation 1).

Figure 2. Structure of Glutathione





GSH synthetase then mediates the reaction of glycine with the cysteine carboxyl of γ -glutamyl cysteine to form the tripeptide γ glutamyl cysteine glycine (equation 2).



The rate limiting step in this biosynthesis is the intracellular pool of cysteine and in the presence of excess cysteine also the activity of the γ -glutamyl-cysteinyl synthetase (γ GCS) enzyme (see Section 1.2.0).

In mammalian cells, GSH exists in at least three, or possibly four, dynamically interchanging forms. Under normal steady state conditions, the majority of GSH exists in the reduced form with concentrations ranging from 0.5 (tissue culture cells) - 10mM (foetal liver), and turnover rates varying from 0.5 (kidney) - 96 hours (mammalian erythrocyte) (Kosower & Kosower, 1978). The GSH contents of some cells are listed in Table 1. Oxidation of reduced GSH, either non-enzymatically or through the action of glutathione peroxidase (GPX), yields GSSG. Levels of GSSG are maintained at low concentrations within the cell, by NADPH-dependent reduction of GSSG by glutathione reductase (GRD) (Douglas, 1987) and efflux from the cell (Sies & Ankerboom, 1984). The normal concentration ranges between 5-50 μ m, but, in some cases, concentrations can reach as high as 100 μ m (see Table 1). A third form of GSH exists as mixed disulphides with both protein and non-protein sulphydryl compounds. It was noted by Guzman Baron (1951) over 35 years ago that the activity of some enzymes appears to be regulated by the formation of protein disulphides, as shown below.

**TABLE 1 GSH, GSSG and GSS-protein (mixed disulphides) content
and turnover in some cells and tissues.**

Cell or tissue	GSH	GSSG	GSS- protein (% of total GSH)	Biological half life GSH (Hours)
erythrocytes (Mammalian)	2.3mM	0.005-0.120mM	-	65-96
Kidney (rat)	2.5mM	0.060mM	20	0.5
Liver (rat)	4.5-6.5mM	0.030-0.470mM	20-50	2-4
Lens (monkey, human, rat, rabbit)	2.6, 3.5, 6.7, 12.0mM	-	10-20	72
Tissue culture and tumour cells	0.8mM, 10-60x 10 ⁻¹⁶ moles/cell	0.012mM	35	6-8
Nervous tissue	2-3.4mM	-	-	72

Abstracted from Kosower & Kosower, 1978.



A potentially significant fourth form of GSH can be found as thiol esters. The evidence for the occurrence and metabolism of these thiol esters is still preliminary. The identification, however, of at least three distinct thiol esterases in human liver perhaps suggest functional significance (Uotila, 1973).

Reduced GSH is characterised by its reactive thiol group and its γ -glutamyl bond where the glutamic acid is γ -linked to the cysteine residue. It has been suggested that this unusual orientation reduces the susceptibility of the glu-cys bond to proteolysis. This is exemplified by the finding that reactions of reduced GSH in mammalian cells can be divided into those involving the γ -glutamyl portion of the tripeptide or those of the sulphydryl moiety. Those involving the sulphydryl moiety can be further categorised into either oxidation reduction reactions (for a review see Ziegler, 1985), or nucleophilic conjugation reactions, in which the reduced sulphydryl reacts with an electrophile to form a thio ether (see Section 1.10.0). The reaction of reduced GSH with reactive electrophiles or peroxides, generated by u.v., radiation, uncoupled mitochondria, or chemicals, can have a variety of consequences. In the majority of cases this reaction is a detoxification pathway, catalysed often by glutathione dependent enzymes; for example, the glutathione S-transferases (GST) (Jakoby, 1978; Chasseaud, 1979; Ketterer, 1982) or GPX (Mills, 1957; O'Brien & Little, 1967; Tappel, 1978) for the peroxides. In chemical carcinogenesis, the production of chemical-induced mutations are reduced, and in chemical toxicity, the interaction with DNA or other critical target sites are prevented. The consequence of these reactions is that intracellular GSH becomes suppressed. The maintenance of intracellular GSH is therefore central to the cellular resistance to chemicals. Consequently, changes in

enzymes involved in the maintenance of GSH levels may occur to try to prevent this decrease.

Apart from its role in the cellular protection from chemical toxins, a wide variety of functions have also been attributed to GSH. Many of these, however, are still speculative. These include the involvement of GSH and GSSG in protein synthesis (Zehavi-Willner *et al*, 1970; Kosower & Kosower, 1973); in oxidative phosphorylation (Painter & Hunter, 1970a, 1970b); in the regulation of the hexose monophosphate pathway (Mills, 1957; Jacob & Jandl, 1966; Eggleston & Krebs, 1974) and in the GSH-dependent biosynthesis of deoxyribonucleotides in bacteria (Holmgren 1979). In conjunction with the GST's, however, GSH is also involved in the biosynthesis of leukotrienes (Bach *et al*, 1984) (see Section 1.10.6), and in melanin (Agrup *et al*, 1977). It has been proposed that GSH in conjunction with γ -glutamyltranspeptidase (γ GT), is involved in the translocation of amino acids across the plasma membrane (Meister 1974), although this is still disputed by Pellifigue *et al* (1976) (see Section 1.4.0). The involvement of GSH in cell division process is also tentative through lack of good studies, but changes in GSH levels during the cell cycle have been described in various species (for a review see Szent-Gyorgyi, 1978). Why GSH levels change during the cell cycle is still at present unclear, but this may involve changes in the different forms of GSH, as described above.

Regulation of GSH levels within cells is thought to occur at the rate limiting step of biosynthesis, ie., at the enzyme γ GCS, where feedback inhibition by high GSH levels, and high levels of intracellular cysteine, stimulate the formation of the GSH precursor (see Section 1.2.0). Exchange of GSH between the cytoplasmic and the smaller mitochondrial pool of GSH, may also influence the levels of GSH (Meredith & Reed, 1982).

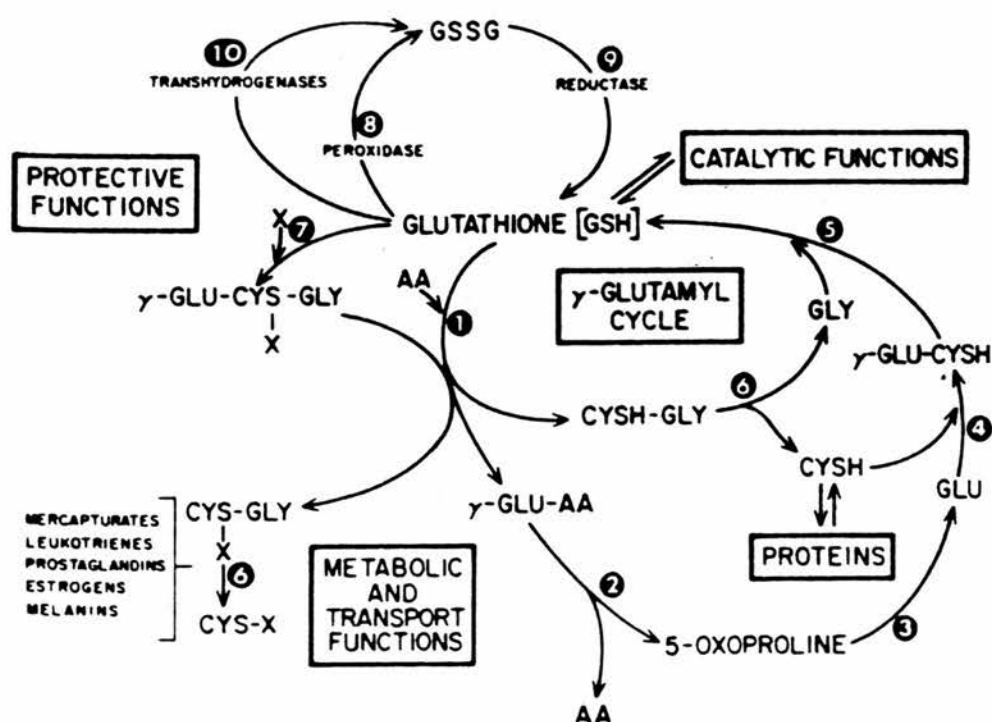
In experimental models, GSH can be regulated by a variety of means. The administration of D-L-buthionine-S-R-sulphoximine (BSO), a potent, specific

inhibitor of γ GCS (Griffith & Meister, 1979a; Dethmers & Meister, 1981), can lead to a reduction in GSH levels. Conversely, cellular GSH levels can be increased by the administration of L-2-oxotazolidine-4-carboxylate (Williamson & Meister, 1981), γ glutamyl cysteine (Anderson & Meister, 1982), or glutathione monoethyl esters (L- γ glutamyl cysteinyl glycylethyl ester) (Puri & Meister, 1983). Such modulation can protect against cytotoxic drug induced damage (Puri & Meister, 1983), and also radiation damage (Wellner et al, 1984). Regulation of GSH levels is, therefore, important, as this may influence the overall response of cells to cytotoxins.

1.1.3 Relationship between reduced GSH and glutathione-dependent enzymes.

Reduced GSH, and the glutathione-dependent enzymes discussed below, are known to be involved in drug detoxification (Chasseaud, 1979; Larsson et al, 1983a; Wolf et al, 1987a, 1987c; Hayes & Wolf, 1988). Apart from this important function, the enzymes are closely linked to the metabolism of GSH. In particular, γ GCS and γ GT are involved in the biosynthesis and breakdown of GSH respectively, forming part of the enzymes of the γ -glutamyl cycle (Figure 3). It is postulated that this cycle in the rat kidney, which consists of a series of six enzymes, functions to transport amino acids across the membrane of the kidney. The scheme shown evolved from the use of specific enzyme inhibitors, and also from studying glutathione regulation in individuals with genetic defects for four of the enzymes : γ -GCS, glutathione synthetase, γ -GT and 5-oxo-L-prolinase (Larsson et al, 1983b). In contrast, glutathione peroxidase (GPX) utilises GSH in the reduction of peroxides to form GSSG, while glutathione reductase (GRD) catalyses the NADPH-dependent reformation of GSH to help maintain the thiol status of the cell. The action of GST's is, effectively, to remove GSH from the cell by catalysing its conjugation to electrophilic compounds, to form mercapturic acids.

Figure 3 An outline of the metabolism and function of glutathione. Enzymes: (1) γ -glutamyltranspeptidase; (2) γ -glutamylcyclotransferase; (3) 5-oxoprolinase; (4) γ -glutamylcysteine synthetase; (5) glutathione synthetase; (6) dipeptidase; (7) glutathione S-transferase; (8) glutathione peroxidase; (9) glutathione reductase; (10) transhydrogenases. AA = amino acids; X = compounds that react with glutathione to yield conjugates, such as those that lead to the formation of mercapturic acids, leukotrienes, and compounds which are involved in other phases of metabolism.



Taken from Meister, 1983.

Consequently, as shown in Figure 3, the glutathione-dependent enzymes under discussion are involved in the metabolism of GSH. Alterations, therefore, in the levels of any of these enzymes may influence not only intracellular GSH concentrations, but also the action of other glutathione-dependent enzymes and ultimately the response of the cell to cytotoxins.

1.2.0 γ -Glutamylcysteinylsynthetase (γ -GCS)

γ GCS (EC 6.3.2.2.) is the rate determining enzyme of GSH biosynthesis in rat liver, catalysing the conjugation of L-glutamate and L-cysteine to form γ -L-glutamylcysteine. Whether this enzyme is the rate limiting step for GSH biosynthesis in cell lines or tumours has not been established.

Most studies on this enzyme have concentrated on its purification and characterisation. γ -GCS has been purified from hog, toad, rat and human foetal liver (Johnston & Block, 1949; Davis *et al*, 1973; Rollins *et al*, 1981); rat kidney (Orlowski & Meister, 1971a, 1971b; Sekura & Meister, 1977a); bovine lens (Rathburn, 1967); and bovine, human, sheep and rat erythrocytes (Wendel, 1974; Majerus, 1974; Board *et al*, 1980; Seelig & Meister, 1984a, 1984b); wheatgerm (Webster & Varner, 1954); E. Coli (Apontowiel & Berends, 1975) and *Proteus mirabilis* (Kumagai *et al*, 1982). The most highly purified preparations are from rat kidney, and rat and sheep erythrocytes. The isolation procedure from rat kidney involved ammonium sulphate precipitation, DE52 column and an ATP-agarose column chromatography. A 37% yield and a purification of 140 fold is reported (Seelig & Meister, 1985).

The molecular weight of the rat kidney enzyme, which comprises two subunits, is about 104,000 (Seelig *et al*, 1984). The heavy subunit has a molecular weight of 73,000, and the light subunit 27,000 (Sekura & Meister, 1977b). ^{The} Heavy subunit contains all the catalytic properties of the isolated enzyme, and, similar to the functional protein, ^{is} feedback inhibited by GSH (Seelig *et al*, 1984). It is thought that the enzyme is synthesised as a 104,000 molecular weight

polypeptide, and processed post translationally to form two subunits, associated by a disulphide bond. This is consistent with Seelig & Meister (1984b), who demonstrated in the kidney enzyme, the presence of one disulphide bond and also two free sulphhydryl groups, of which only one is involved at the active site. At the molecular level, a gene for γ -GCS from *E. Coli* has been cloned and expressed in mammalian cells (Gushima *et al*, 1983), the human equivalent as yet, has not been described.

The tissue distribution of γ GCS, especially in human tissues, has not been studied in detail. Indications that it is found in all human tissues, however, are based on the use of skin fibroblast biopsies for phenotyping for this enzyme. Since individuals who lack this enzyme survive, this indicates that the function of this enzyme, in the biosynthesis and regulation of GSH levels, may not be essential for life.

γ GCS is induced in rat kidneys by selenium supplementation, through a mechanism that involves de novo protein synthesis (Chung & Maines, 1981). Conversely, Hill & Burk (1982) have also shown that selenium deficiency induces the activity of this enzyme. The expression of the *E. Coli* gene for γ GCS in mammalian cells will help to clarify these conflicting reports. The binding specificity of the γ -glutamate to the active site allows further regulation of this enzyme by the feedback competitive inhibition site by GSH (K_i 2.3mM) (Davis *et al*, 1973; Rickman & Meister, 1975). The availability of the precursor, cysteine (Thor *et al*, 1978; Vina *et al*, 1978; Beatty & Reed, 1980), also allows regulation of the enzyme. The K_m of γ -GCS, for its substrates cysteine and glutamate, are 0.36mM and 2mM respectively (Rickman & Meister, 1975).

γ GCS can, however, also be inhibited by certain analogues of its substrate L-glutamate, such as D-glutamate (Sekura & Meister, 1977a), cysteamine (Seelig & Meister, 1982), chloroketones (Beamer *et al*, 1980), and several analogues of methionine sulfoximine e.g. propionine and buthionine (Griffith & Meister, 1979a;

Griffith, 1982). The potency of inhibition is in the increasing order: methionine; propionine and buthionine (Griffith & Meister, 1979a; Griffith, 1982). Structure of buthionine sulfoximine is shown in Figure 4. The role of this inhibitor in depletion of GSH and modulation of chemotherapy is discussed later.

1.3.0 Glutathione Synthetase

Glutathione synthetase (EC 6.2.3.2) is the second enzyme involved in the biosynthesis of reduced GSH, catalysing the conjugation of glycine to glutamyl cysteine to form the tripeptide glutathione. Very little work has been carried out on this enzyme, but it has been purified from a variety of sources, including human erythrocytes (Majerus *et al.*, 1974).

The enzyme from yeast, bovine erythrocytes, and rat kidney, exhibit molecular weights of about 120,000, while a somewhat higher value was reported for the human erythrocyte enzyme (150,000). The bovine and rat kidney enzymes dissociate in the presence of SDS to a species of 60,000 molecular weight, suggesting the enzyme is composed of two identical subunits. Interestingly, a disease exists where patients who have severe glutathione synthetase deficiency (5-oxoprolinuria) have symptoms that include mental retardation and excretion of 5-oxoproline (Larsson & Hagenfeldt, 1983).

1.4.0 γ -Glutamyltranspeptidase (γ GT)

γ GT (EC 2.3.22) is an extracellular membrane bound protein that catalyses the initial step in the degradation of reduced GSH and its derivatives (Puri & Meister 1983). The γ -glutamyl (γ -glu) moiety of the tripeptide is transferred to an acceptor molecule, which can be an amino acid (equation 5) (or dipeptide) or GSH, itself (equation 6) producing a γ glutamyl conjugate. If the nucleophile is water (equation 7), hydrolysis occurs, resulting in the formation of glutamate. These three general types of reactions are indicated below:-

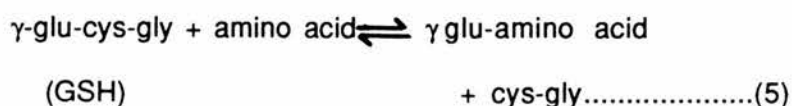
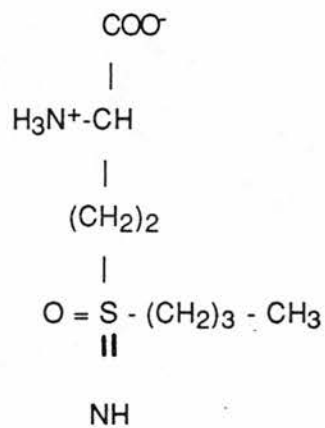
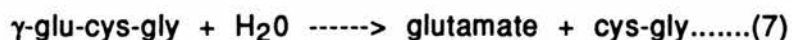


Figure 4 Structure of Buthionine Sulphoximine.





γ GT has been purified from different tissues from various species (Revel & Ball, 1959), but, so far, the kidney has the highest activity (Goldbarg et al, 1960). Rat renal γ -GT is a glycoprotein containing two distinct subunits. The renal enzyme is attached to the brush border membrane via a short NH₂ -terminal hydrophobic region of the larger subunit, molecular weight 51,000. The smaller subunit (molecular weight 22,000), contains the active site which is involved in the formation of covalent γ -glutamyl-enzyme intermediate. Pulse chase studies into the biosynthesis, processing, and assembly of the heterodimeric γ GT enzyme indicates that γ GT is initially synthesised as a glycosylated, single chain precursor (molecular weight 78,000), which is subsequently cleared into the two polypeptide subunits (Tate & Meister 1977; Nash & Tate 1982; 1984). The isolation of γ -GT cDNA clones (Laperche et al, 1986) and the generation of E. Coli K12 mutants lacking γ -GT (Suzuki et al 1987), will greatly facilitate studies into the processing of γ GT, and also its regulation and function. The human renal γ -GT has also been recently cloned, and mapped to chromosome 22 (Bulle et al, 1987).

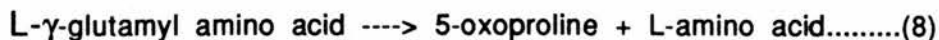
γ -GT has a wide tissue distribution, including the kidney, the biliary epithelium, the lining of the small intestine, and other tissues which have a secretory function, such as pancreas and seminal vesicles (Tate 1980; Hanigan & Pitot, 1985). The proposed functions of this enzyme may be, in fact, related to this distribution profile. The reaction catalysed by γ -GT is, for instance, important in the γ -glutamyl cycle (as mentioned in Section 1.1.3), a metabolic pathway involved in the enzymatic synthesis and breakdown of GSH, and constitutes one of the possible mechanisms for the transport of amino acids into certain cell types (Orlowski & Meister, 1970; Meister & Tate, 1976; Meister, 1981; Meister & Anderson, 1983). This function still remains controversial, however, as Pellefigue et al (1976) demonstrated that amino acid uptake still

occurred in a γ -GT deficient human fibroblast cell line. γ -GT is also involved in the formation of mercapturic acids from thioethers, with the removal of the γ -glutamyl moiety. This type of reaction also occurs in endogenous metabolism; for example, in the formation of leukotriene D. This is the slow reacting substance of anaphylaxis, and is synthesised from leukotriene A, which reacts with glutathione to form leukotriene C. Subsequent removal of the γ -glutamyl moiety of leukotriene C by γ -GT yields leukotriene D (Orning et al 1980). Analogous reactions involving γ -GT are also found in the metabolism of prostaglandins (Cagen et al 1976), steroids (Kuss, 1969), and melanins (Agrup et al, 1977).

Cellular levels of γ -GT can be modulated by a variety of factors; for example, in transformation of rat hepatocytes with active ras oncogenes, or the in vivo administration of aflatoxin B₁ (Manson, personal communication) glucocorticoids (Billion et al, 1980; Barouki et al, 1982) and the antioxidant ethoxyquin (Manson et al, 1987) all lead to the elevation of γ -GT levels. The in vitro γ -GT activity can be inhibited reversibly by L-serine in the presence of borate (Revel & Ball, 1959), and L- γ -glutamyl-(O-carbonyl) phenylhydrazide (L-OC). In the latter case, the apparent K_i value is 8.5 μ M, compared with 1.45 mM for L-serine plus borate (Griffith & Meister, 1979b). γ -GT is irreversibly inhibited in vitro using glutamine antagonists L-(α -S,5 S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) (Griffith et al, 1980). The administration of L-OC or AT-125 to mice leads to glutathionuria (Griffith & Meister 1979b), as well as the urinary excretion of γ -glutamylcysteine and cysteine moieties (Griffith et al, 1980). This indicates that γ -GT has a function in the metabolism and/or transport of these sulphur containing compounds, as well as glutathione (Griffith et al, 1980).

1.5.0 γ -Glutamylcyclotransferase

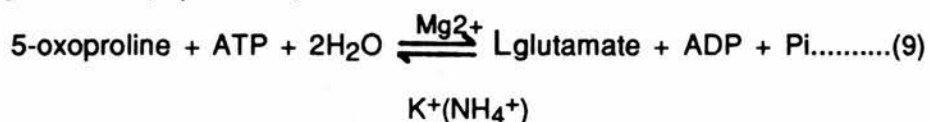
γ -Glutamylcyclotransferase (EC 2.3.2.4) catalyses the conversion of γ -glutamyl amino acids to 5-oxoproline and the corresponding amino acid (equation 8) (Meister & Anderson, 1983).



Although the enzyme has been purified from various sources, including human erythrocytes (Board *et al*, 1978), the purified enzyme is unstable and, consequently, very little work has been carried out on it. The molecular weight has been estimated at 27,000 for the rat kidney polypeptide (Meister, 1985). The enzyme forms part of the γ -glutamyl cycle (see Figure 3).

1.6.0 5-Oxo-L-prolinase

5-oxo-L-prolinase (EC 3.5.2.9) catalyses the ATP-dependent cleavage of 5-oxoproline to glutamate (equation 9)



This reaction seems to be the only one presently known in which cleavage of ATP is required for the cleavage of a peptide bond. ATP required is mandatory because of the unusual stability of the internal bond of 5-oxoproline. 5-Oxoprolinase has been purified from rat kidney and is a homodimer with a subunit molecular weight of 162,500 (Meister *et al*, 1985). The enzyme is found in a variety of mammalian tissues, including kidney, spleen and liver (van der Werf *et al*, 1971). Again, this enzyme has not been studied in detail. Patients with deficiencies in this enzyme have also been found (Larsson *et al*, 1983b).

1.7.0 Dipeptidase

The dipeptidase cleaves the cysteinyl-glycine bond to release cysteine and glycine. This peptidase has been purified from rat kidney (Stetson, 1975) and is found to be widely distributed. Apart from the fact that it is involved in the γ -

glutamyl cycle (see Figure 3), and in the formation of mercapturic acids, it has not been well characterised.

1.8.0 Glutathione reductase (GRD)

GRD (EC 1.6.4.2) is a flavoprotein which catalyses the NADPH-dependent reduction of glutathione disulphide (GSSG) to glutathione (GSH) (equation 10) (Racker, 1955).

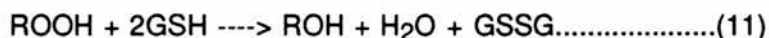


GRD has been purified from various sources, including rat liver (Calberg *et al*, 1981), yeast (Calberg & Mannervik, 1977) porcine and human erythrocytes (Boggaram *et al*, 1979). The enzyme exists as a homodimer with a molecular weight of 100,000 and contains one FAD molecule per subunit. The amino acid sequence (Krauth-Siegal *et al*, 1982) and high resolution X-ray diffraction data of the native human GRD (Thieme *et al*, 1981; Schulz *et al*, 1978; 1982) and the enzyme:coenzyme/enzyme:substrate complexes (Pai & Schulz, 1983) has led to detailed information on the reaction mechanism (Pai & Schulz, 1983). The GRD gene for *E. Coli* has been cloned and sequenced (Greer & Perham, 1986) and is highly homologous to the human GRD amino acid sequence (Perham, 1987). One of the differences is, however, the substitution of a histidine at position 219 at the catalytic site in the human enzyme for a lysine in the *E. Coli* enzyme. More significant is the cysteine at position 90 in the human enzyme, which forms an unusual intersubunit disulphide bridge (Thieme *et al*, 1981), which, in the *E. Coli* protein, is replaced by threonine, resulting in a non covalently linked dimer. As there is homology between the *E. Coli* GRD protein and the human GRD protein, site directed mutagenesis of the *E. Coli* gene and the use of Fuchs' GRD deficient *E. Coli* mutants (Fuchs *et al*, 1983), makes it possible to test some of the predictions made by Pai & Schulz (1983) concerning the reaction mechanism, regulation and function of the enzyme.

GRD is found in almost all tissues and in all cells (Calberg *et al*, 1981), and functions to maintain GSH levels in the reduced state. The enzyme has been shown to be induced by compounds such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Kumar *et al*, 1984), diethylnitrosamine (Pinto & Bartley, 1973), and metals such as selenium (Chung & Maines, 1981). Inhibition of GRD *in vivo* in erythrocytes of patients receiving chemotherapy with 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) was first reported by Frischer & Ahmad (1977). Further studies by Babson & Reed (1978) demonstrated that inactivation of rat GRD only occurs when the enzyme is in a reduced state (ie. in the presence of NADPH) and two thiol groups are present at the active site. The inactivation of catalytic activity appears to occur as a thio carbamate adduct forms between the isocyanate metabolite of the nitrosourea and probably a distal thiol group at the active site of the enzyme (Babson & Reed, 1978). This was confirmed by X-ray diffraction studies of the enzyme inhibitor complex (Bilzer *et al*, 1984).

1.9.0 **Glutathione Peroxidase (GPX)**

Glutathione peroxidases catalyse the reduction of hydroperoxides (ROOH) by GSH (Mills, 1957; Wendel, 1980; Flohe, 1982) Equation 11.



R can be aliphatic, an aromatic organic group or hydrogen. The products generated are an alcohol, water and GSSG. Regeneration of GSH from GSSG is catalysed by GRD, as previously mentioned in Section 1.8.0.

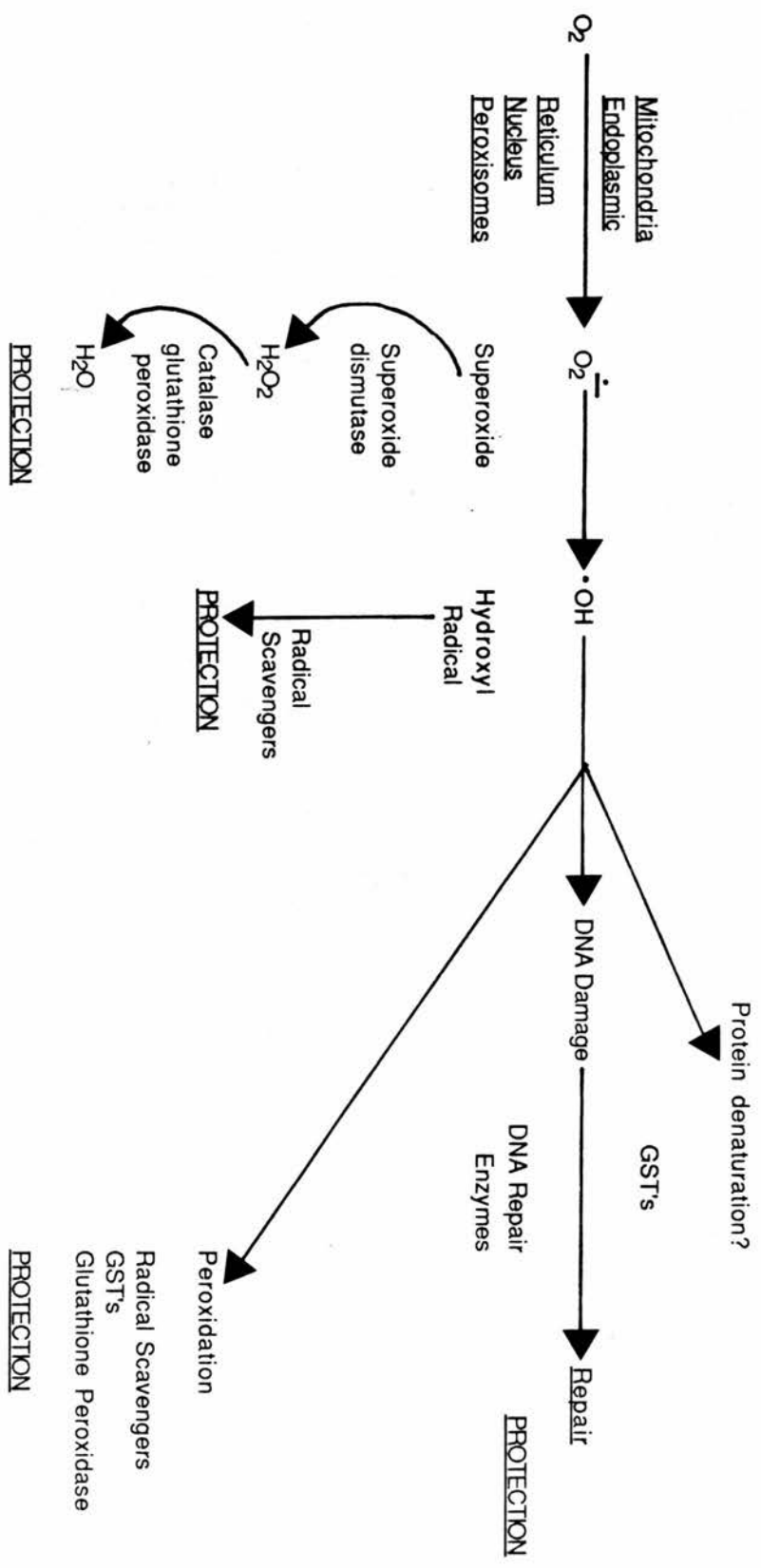
Two major types of GPX have been identified. A selenoenzyme containing selenium in the form of covalently bound selenocysteine at the active site (Rotruck, 1973), and a non-selenium containing protein with negligible activity towards hydrogen peroxide (H₂O₂) (Lawrence & Burk, 1976; Prohaska & Gunther, 1976; Hunter *et al*, 1976). This activity has been associated with the glutathione S-transferase family of enzymes (Prohaska & Gunther, 1977). Discussion of the latter enzymes is described also in Section 1.10.0.

The selenium-dependent glutathione peroxidase (GPX) (EC 1.11.19) was discovered in erythrocytes by Mills (Mills, 1957) and has since been purified from a variety of sources, including rat liver and lung (Nakamura et al, 1974; Chiu et al, 1976), bovine erythrocytes (Ladenstein et al, 1979), and human placenta and erythrocytes (Awasthi et al, 1975; 1979). The bovine enzyme is a tetramer with a molecular weight of approximately 80,000 (Flohe et al, 1971). Recently, however, selenium-dependent GPX has been purified from human plasma (Kakahashi et al, 1987). The enzyme, like the erythrocyte form, is tetrameric and contains 4 atoms of selenium/mole, but, unlike the erythrocyte protein, is glycosylated, and subsequently, has a molecular weight of 100,000. The structure of the bovine erythrocyte selenium-dependent GPX has been determined by X-ray diffraction analysis (Epp et al, 1983). Its amino acid sequence has also been elucidated (Flohe et al, 1984).

At the molecular level, the entire mouse selenium-dependent GPX gene has been cloned and sequenced (Chambers et al, 1986). Evidence from this work indicated that the selenocysteine at the active site of the enzyme is encoded by the stop codon TGA. Sequence of a cDNA clone coding for the human enzyme confirmed this finding (Mullenbach et al, 1987). The human selenium-dependent GPX gene has also been mapped to a region on chromosome 3 (3p13 ----> 3q12) by use of mouse and hamster somatic cell hybrids (Johannsmann et al, 1981; Wijnen et al, 1978).

The subcellular localisation of selenium-dependent GPX is important in its function, as catalase can also metabolise H_2O_2 as substrate (see Figure 5). The relationship between catalase and selenium-dependent GPX has been studied (Wendel, 1980). In hepatocytes, catalase is primarily localised in the peroxisomes, while selenium-dependent GPX is found both in the cytosol and mitochondrial matrix. The two enzymes, therefore, appear to have complimentary localisations and functions in the cell. GPX activity has been demonstrated in all mammalian tissues (Flohe, 1982). The selenium-dependent GPX is responsible for

Figure 5. Diagrammatic representation of some enzymes involved in protection against oxygen toxicity.



most of the activity, but in certain species, such as guinea-pig liver, the selenium-dependent enzyme is absent (Lawrence & Burk, 1978). The ratio between the selenium-dependent and non-selenium dependent activity may vary, not just between animal species, but also from tissue to tissue within the same species (Carmagnol et al, 1983). The factors involved in the tissue specific expression of selenium and non-selenium-dependent GPX is unclear. It is known, however, that selenium concentrations can influence the relative expression of these enzymes (Masukawa et al, 1983).

The GPX enzyme functions to protect against toxic oxygen products generated by radiation, U.V. light, and uncoupled mitochondria (Figure 5). In particular, both forms of GPX catalyse the reduction of lipid hydroperoxides (Tan et al, 1984), and of peroxidised DNA (Christophersen, 1969; Tan et al, 1986; Ketterer et al, 1987). It is therefore postulated that GPX protects cells from oxidative damage through the protection of the cell membrane against lipid peroxidation (reviewed by Flohe, 1982), and in the protection of macromolecules from oxidative damage (Flohe & Zimmerman, 1970; Tappel, 1974; Combs et al, 1975). Mitochondrial selenium-dependent GPX is also, however, involved in the regulation of mitochondrial substrate oxidations (Sies & Moss, 1978) and protection of mitochondrial enzymes and DNA against lipid peroxidative damage (Timcenko-Youssef et al, 1985). Apart from the effect of selenium, no other forms of regulation of GPX activity have been described. The use of GPX inhibitors may, therefore, have a potential use in both anticancer therapy and in the regulation of mitochondrial activity. Misonidazole (1,2-nitro-1-imadazolyl-3-methoxy-2-propanol) a radiosensitiser, has shown to inhibit both forms of GPX in vitro and in vivo (Kumar & Weiss, 1986) and may be of therapeutic use, as specific inhibitors for selenium-dependent GPX have not yet been reported.

1.10.0 **Glutathione S-transferases (GST's)**

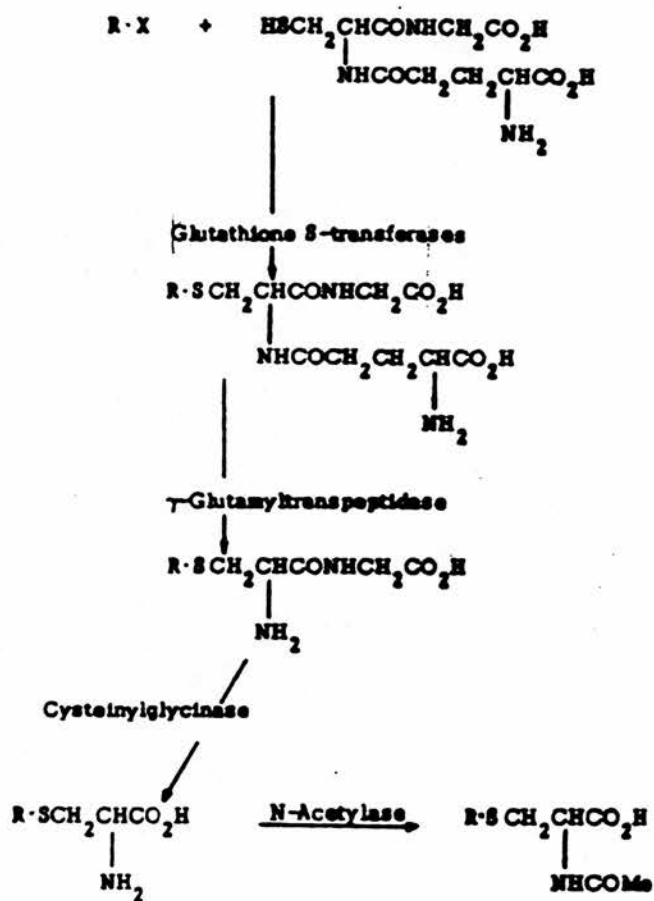
As a lot of the work described in this thesis centres around the GST's, the properties of these enzymes will be discussed in some detail.

1.10.1 Introduction

The glutathione S-transferases (GST, EC 2.5.1.18) are a multigene family of dimeric proteins, which catalyse the conjugation of genotoxic and cytotoxic xenobiotic electrophiles to GSH. Evidence that mammalian GST's are important in xenobiotic metabolism has come from pharmacological and biochemical studies, showing the GST's catalyse the first step in mercapturic acid formation (Booth et al, 1961) (Figure 6). These enzymes, however, also possess GSH-dependent isomerase activity (Benson et al, 1977) and non-selenium-dependent GPX activity (Prohaska & Ganther, 1977), which are also of importance in the biological function of these proteins (see Section 1.10.6).

GST was first identified by Booth et al (1961) as "an enzyme from rat liver catalysing conjugations with glutathione", using a number of electrophilic compounds, including 1,2-dichloro-4-nitrobenzene, benzyl chloride and bromoethane. Multiple forms of GST was postulated, with each form being specific for certain functional groups; for example, aryl, alkyl and epoxy groups (Grover & Sims, 1964; Boyland & Williams, 1965). This was later found not to be the case. In 1967, Ketterer et al (1967), isolated a carcinogen binding protein, which was trivially named ligandin (Ketterer & Beale, 1971). It was not, however, until 1974, following the discovery that 1-chloro-2,4-dinitrobenzene (CDNB) was a substrate for essentially all GST's (Clark et al, 1973), that Jakoby and co-workers identified ligandin as a member of this group of proteins (Habig et al, 1974a). GST activity has now been detected in almost all living organisms ranging from bacteria, yeast and plants to fish, birds and all mammalian species. (Clark et al, 1973; Pabst et al, 1973; Kamisaka et al, 1975; Asaoka et al, 1977;

Figure 6. Pathway of mercapturic acid biosynthesis.
(Taken from Chasseaud, 1976).
R - X electrophilic compound



Benson et al, 1978; Smith et al, 1980; Irwin et al, 1980; Yeung & Gidari, 1980; Saneto et al, 1980; Mozer et al, 1983).

1.10.2. Characterisation of GST proteins.

The purification of GST's revolutionised the characterisation of these enzymes. Purification is carried out using conventional techniques. However, major advances were achieved using affinity chromatography and chromatofocussing steps. Affinity resins include immobilised GSH coupled through the sulphur atom (Vander jagt et al, 1985), or immobilised S-hexyl glutathione coupled through the α -amino group of the glutamyl residue (Guthenberg & Mannervik, 1979). Recent application of high performance liquid chromatography, and fast performance liquid chromatography (h.p.l.c. and f.p.l.c.), (Hayes et al, 1987b), allows quicker and easy resolution of the GST subunits from smaller samples. GST's have now been purified from a variety of mammalian sources, including rat liver (Hayes et al, 1981; Beale et al, 1983; Hayes, 1984; 1986; McCusker & Mantle, 1987); kidney (Guthenberg et al, 1979) and brain (Singh et al, 1987); mouse liver (Lee et al, 1981; Warholm et al, 1986; Hayes et al, 1987b; McLellan & Hayes, 1987); human lung (Koskelo et al, 1981); liver (Stockman et al, 1985; Stockman et al, 1987); placenta (Guthenberg et al, 1985; erythrocytes (Marcus et al 1978; Stockman et al, 1985); kidney (Koskelo & Icen, 1984); skin (Del Boccio et al, 1987) and thyroid (Del Boccio et al, 1986). Purified GST's have also been obtained from other diverse sources such as corn (Timmerman & Tu, 1987) and drosophila (Cochrane et al, 1987). This list is not meant to be comprehensive, but illustrates the wide range of tissues and sources from which GST's have been purified. As GST's were isolated by many different groups, it is important to establish a nomenclature to allow comparison of forms.

1.10.3 Nomenclature.

Originally GST's were described on the basis of their function, for example, GSH-S-aryl chloride transferase (Grover & Sims, 1964), GSH-S-

epoxide transferase (Boyland & Williams, 1965), GSH-S-alkyl transferase (Booth et al., 1961), GST-S-aryl transferase (Boyland & Chasseaud, 1967) etc. This was, however, found unsuitable, as certain enzyme substrates were metabolised by more than one protein, and vice versa the best example being 1,4-dinitrobenzene (CDNB) which is a substrate for essentially all GST's (Clark et al., 1973). Using this substrate as a marker, GST's were isolated from rat liver cytosol and named alphabetically, in reverse order of their elution from CM-Cellulose (Jakoby et al., 1976). Bass et al. (1977) showed that the three major classes of GST subunits from rat liver had different apparent molecular weights on sodium dodecyl sulphate (SDS) polyacrylamide gels. These subunits were classified as Ya, Yb, and Yc, in order of their increasing molecular weight. Y represented the second peak of elution from the column on which they were initially purified, ^{was the first peak, Y the second and Z the third.} i.e. X, ~~Y~~ Z. This nomenclature has been further extended by Hayes et al. (1984; 1986) and Beale et al. (1983). Mannervik and Jensen (1982) described a nomenclature similar to that of Bass et al. (1977) and Hayes et al. (1984; 1986) where each subunit was given a single letter of the alphabet. Since the GST's were shown to be a large complex multigene family of enzymes (Ketterer et al., 1984), a numerical system was devised to allow for expansion of the nomenclature to accommodate new GST's found (Jakoby et al., 1984). Table 2 illustrates the nomenclatures used for the rat GST's and the comparison between rat, mouse, and human GST's.

The cloning and primary amino acid sequence, together with structural homology based on antibody reactivity, has allowed classification of the cytosolic GST's into three separate gene classes I, II, and III (Tu et al., 1987; Hayes & Mantle, 1986b; Mannervik, 1985), ~~termed~~ termed alpha, mu and pi (Mannervik et al., 1985) respectively. A high degree of sequence homology is found between GST's within a gene family, and also between homologous families between species (Ketterer, 1986). Group IV contains the microsomal GST enzyme which, on the

basis of immunoblotting, shows structural homology between all species studied so far (Morgenstern, 1987; Hayes et al, 1987a; McLellan & Hayes, 1987).

In the context of this thesis, the human GST's will be referred to as alpha (α - ϵ , B₁), mu (μ , ψ , ϕ) and pi class (P, λ , π). As suggested by their isoelectric points on chromatofocussing gels, these classes are equivalent to basic, neutral, and acidic GST's. The overlap of the isoelectric points for proteins in the neutral group with those of the acidic protein (Table 2) does not allow the use of these terms to classify the human GST's. The homologous proteins in rat and mice are also given this terminology, but are also referred to by their subunit classification (Table 2).

Identification of different GST subunits purified from different species into one of the three main groups can be obtained by the use of specific substrates and inhibitors (see Tables 3, 4). In general, cumene hydroperoxide is a diagnostic substrate for alpha class GST's, while trans-4-phenyl-3-buten-2-one is for the mu class, and ethacrynic acid for pi class (Mannervik, 1985). Class IV, the microsomal GST, is unusual, in that the activity of this enzyme towards CDNB is activated 15 fold upon treatment with N-ethylmaleimide (Morgenstern et al, 1987); however this group has high activity towards the substrates such as hexachloro-1,3-butadiene (Wolf et al, 1984).

1.10.4 Tissue Distribution.

The tissue distribution of GST's is often quantitatively and qualitatively characteristic of a tissue. In this regard, rat liver has been most studied, and at least 10 different isoenzymes have been found (Mannervik, 1985). Differences in the distribution of GST subunits even within a tissue, were observed, through immunocytochemical staining, in both kidney (Boyce et al, 1987) and liver (Redick et al, 1982; Boyce et al, 1987). The tissue distribution of the GST's will be described according to the gene family.

TABLE 2 NOMENCLATURE FOR RAT, MOUSE AND HUMAN GSTS

RAT						MOUSE				HUMAN			
Family	Jakoby	Beale	Bass	Mannervik & Jensen*	Subunit Size** (x10 ⁻³)	Isoelectric Point*** (PI)	Nomenclature	Subunit Size ^a (x10 ⁻³)	Isoelectric Point ^a (PI)	Nomenclature	Subunit Size ^b (x10 ⁻³)	Isoelectric Point ^a (PI)	
I	1 - 1	B ₁	YaYa	L ₂	25.5	9.1	Ya	26.0	9.2	B ₁ B ₁ (ε)	26.0	8.9	
	1 - 2	B ₂	YaYc	BL		9.1				B ₁ B ₂ (δ)		8.75	
	2 - 2	AA	YcYc	B ₂	27.5	9.0	MI	25.0	9.7	B ₂ B ₂ (γ)	26.0	8.4	
	8 - 8	K	YkYk		25.0								
	3 - 3	A	Yb ₁ Yb ₁	A ₂	26.3	8.4	MIII Yb F ₃	27.0	7.8-8.2	μ	26.7	6.1	
	3 - 4	C	Yb ₁ Yb ₂	AC		7.4				ψ	26.5	5.5	
	4 - 4	D	Yb ₂ Yb ₂	C ₂	26.3	6.7				φ	26.5	4.8	
	5 - 5					7.0							
	6 - 6		YnYn		26.0	5.3				π	24.8	4.8	
	7 - 7		YfYf		24.5	6.8	MII Yf F ₁ /F ₂	24.5	8.6	λ	24.8	4.8	
IV	Microsomal ^c				17.0	ND	Microsomal ^d	17.0	ND	Microsomal ^e	17.0	ND	

Note: N.D. is not determined; N.K., not known. + = Jakoby *et al.*, 1984; ++ = Beale *et al.*, 1983; +++ = Y nomenclature based on Ya, Yb, Yc designation of Bass *et al.*, 1983 and Hayes, 1984; 1986. * = Mannervik & Jansson, 1982; ** = Hayes & Mantle, 1986b; *** = Ketterer, 1986.

a = based on information from Hayes *et al.*, 1987b; Mclellan & Hayes, 1987; Warholm *et al.*, 1986, and Lee *et al.*, 1981.

b = based on information from Board & Webb, 1987; Del Boccio *et al.*, 1987; Hayes *et al.*, 1987a, and Stockman *et al.*, 1987.

c = Morgenstern *et al.*, 1987; d = Mclellan & Hayes, 1987; e = Hayes *et al.*, 1987a.

TABLE 3 SUBSTRATE SPECIFICITIES OF RAT AND HUMAN GST'S
RELATIVE SPECIFIC ACTIVITY (%)^a

Substrate	RAT										HUMAN		
	1-1	1-2	2-2	3-3	3-4	4-4	6-6	7-7			Basic (α-ε)	Neutral (μ)	Acidic (π)
1-Chloro-2,4-dinitrobenzene	100 (33)	100 (28)	100 (19)	100 (38)	100 (28)	100 (18)	100 (190)	100 (20)			100 (64)	100 (187)	100 (105)
1,2-Dichloro,4-nitrobenzene	<0.2	<0.2	0.2	<u>1.0</u>	9	1	1.5	ND			0.05-0.10	0.02	<u>0.10</u>
Bromosulphophthalein	ND	ND	ND	<u>2</u>	1	0.2	ND	NC			0.002-0.02	<0.001	<0.002
Ethacrynic acid	0.4	3	<u>7</u>	0.3	1	3	0.03	<u>2.0</u>			0.03-0.07	0.04	<u>0.82</u>
Trans-4-phenyl-3-buten-2-one	ND	ND	ND	0.2	2	<u>9</u>	0.01	0.1			0.002-0.003	<u>0.19</u>	0.009
Styrene-7-8 oxide	NC	NC	NC	NC	NC	NC	NC	NC			0.03	<u>1.39</u>	0.07
Cumene hydroperoxide	8	14	<u>4.0</u>	1.4	2	7	0.1	0.05			<u>16.6</u>	0.34	0.03
Δ ⁵ -Androstene-3,17-dione	<u>1.3</u>	8	2	0.05	0.03	0.01	ND	0.005			<u>12.5</u>	0.06	0.009
p-Nitrophenyl acetate	2	1.3	0.4	2	1.3	1	0.1	NC			<u>0.28</u>	0.12	0.18

a = table taken from Mannervik, 1985 and Ketterer, 1986; b = values given as percentages of the specific activities determined with 1,chloro-2,4-dinitrobenzene as substrate (given in parenthesis as μmol/min/mg); ND = no detectable activity under assay conditions used; NC = not carried out. Underlined values are the isoenzymes with highest activities towards the substrate.

TABLE 4 INHIBITION CHARACTERISTICS FOR RAT AND HUMAN GST'S

I₅₀ value (μM)

Inhibitor	RAT						HUMAN		
	1-1	2-2	3-3	4-4	6-6		Basic (α-ε)	Neutral (μ)	Acidic (π)
Cibacron Blue	0.6	20	0.25	0.1	<u>0.02</u>		5	<u>0.05</u>	0.5
Tributyltin acetate	2	<u>0.5</u>	1	2	10		<u>0.1</u>	0.5	4
Triphenyltin bromide	350	3	<u>1</u>	70	100		10	5	6
Triphenyltin chloride	0.5	30	<u>0.1</u>	0.2	30		<u>0.25</u>	0.5	>10
Bromosulphthalein	2	200	10	<u>0.5</u>	1		75	<u>2</u>	100
Hematin	<u>0.05</u>	2	1	1	0.3		<u>0.5</u>	1	5
5-(p-Bromobenzyl) glutathione	50	<u>2</u>	25	6	25		4	<u>1</u>	4

Taken from Mannervik, 1985. The values underlined indicate the most sensitive enzyme activity to the inhibitor.

(a) Alpha class

In rat tissues, many GST subunits have been identified in this gene family, and are classified as Ya, Yc, Yk subunits etc. The rat Yc GST subunit has been described in most tissues, including liver, kidney, lung, heart and testis. Ya, in contrast, is found mainly in the liver, kidney, and small intestine. The Yk GST subunit is found in most tissues, but at highest concentration in the lung (Hayes & Mantle, 1986b). The mouse liver contains mainly Ya subunits (Hayes et al, 1987b; McLellan & Hayes, 1987). In humans, alpha class GST's are found in the liver (Stockman et al, 1985; 1987), skin (Del Boccio et al, 1987), kidney, and testis (Sherman et al, 1983).

(b) Mu class

In rat tissues, several subunits in this class have been identified; for example, Yb₁, Yb₂, Yn. The subunits Yb₁ and Yb₂ are found in most tissues. The Yb₁ subunit is, however, absent from the kidney. Another mu class enzyme, Yn, is also found in the heart and spleen, and is the major form in the testis. In human tissues, mu class proteins have been identified in the adrenal gland, testis, liver and lung (Sherman, 1983; Hayes et al, 1987b; Carmichael et al 1988), and is polymorphic in man (see Section 1.10.7).

(c) Pi class

Most evidence indicates that there is only one protein in this GST group. This pi class GST is present in almost all rat tissues, with the highest levels in the colon, lung, and spleen (Hayes & Mantle, 1986b). This protein is also detected at low levels in rat and human liver but, interestingly, is expressed in a sex specific manner in the mouse, with male liver containing high levels of this subunit (McLellan & Hayes, 1987). In man, this polypeptide also seems to be widely distributed throughout tissues (for a review see Mannervik, 1985), and is present at high levels in many tissues, although individuals may lack pi GST subunit in the liver (Hayes et al, 1987c). The difference in the tissue distribution as described,

and substrate specificities (see Section 1.10.6), can, therefore, influence the response of a tissue to a particular cytotoxic compound.

1.10.5 Comparison of GST between species.

GST's are found in all species. However, they have been best characterised in rat, man, and mouse. Structural homologous forms are found to exist between all these three species (see Table 2). Studies in other species, such as guinea pig, rabbit, and hamster, also identified alpha class Yc and mu class Yb GST's present in the liver (Hayes & Mantle, 1986a; Igarashi *et al*, 1986).

Further investigation into comparison of GST's between species can now be carried out using molecular analysis such as 'Northern' and 'Southern' blots. Comparison of GST cDNA sequences would provide information not only about the sequence homologies between species but also in determining functional regions of the sequence. In particular, cDNA clones for GST's are available for rat liver alpha class [Ya (Lai *et al*, 1984; Pickett *et al*, 1984) Yc (Tu *et al*, 1984)]; mu [Yb (Ding *et al*, 1985)] and pi class (Sugioka *et al*, 1985) subunits; human alpha class (Tu & Qian, 1986) and human pi class (Kano *et al*, 1987) subunits; mouse alpha class [Ya] subunit (Daniel *et al*, 1987); corn GST_i (Shah *et al*, 1986), corn GST_{iiia} (Moore *et al*, 1986) and trematode *shistosoma japonicum* (Smith *et al*, 1986). Genomic clones are also available for rat Ya, Yb, and Yf (Telakowski-Hopkins *et al*, 1986; Masuda *et al*, 1986). In mouse GST Ya and Yf have been mapped to chromosome 9 and 5 respectively (Czosnek *et al*, 1984; Lalley & McKusick, 1985), human pi has been mapped to chromosome 11 (Suzuki & Board, 1984), and alpha to chromosome 6 (Board & Webb, 1987). Rat pi has been localised to chromosome 1 q43-q51 (Masuda *et al*, 1986).

1.10.6 Substrate Specificity and Biological Function of GST's.

GST's are a multifunctional group of enzymes which carry out a wide variety of physiological roles within an organism. These polypeptides can act as enzymes or as binding proteins. In the latter case, GST can bind a variety of

compounds such as haem, bilirubin, and bile acids (Hayes & Mantle, 1986c), as well as exogenous carcinogenic polycyclic aromatic hydrocarbons; for example, benzo(a)pyrene (Coles et al, 1967; Awasthi & Singh, 1987). Alpha class Ya and Yc GST subunits are specifically associated with the binding of benzo(a)pyrene, both in vivo and in vitro, in lung and liver tissues (Awasthi & Singh, 1987). It has been proposed that this binding to GST's represents a detoxification reaction.

The enzymatic function of GST involves the metabolism of both endogenous and exogenous substrates (xenobiotics), indicating functions in intermediary metabolism as well as detoxification.

a) Endogenous substrates

Although GST will catalyse the conjugation of many endogenous compounds, in many cases the physiological significance of these reactions is not clear.

GST's catalyse the GSH-dependent isomerisation of double bonds; for example, the conversion of Δ^5 -3-ketosteroids to the corresponding α,β -unsaturated Δ^4 -3-ketosteroids (Benson et al, 1977). This activity, which may be important in prostaglandin biosynthesis (Christ-Hazelhof & Nutgeren, 1979; Meyer & Ketterer, 1987), is so far restricted only to the Ya subunits. The formation in vivo and in vitro of some GSH conjugates of steroids, suggests that the reactive intermediates of 17β -estradiol (Marks & Hecker, 1969) and 2-hydroxy- 17β -estradiol (Elce & Harris, 1971), are physiological substrates for GST's. The possibility that quinones are also substrates for GST's has long been recognised both for oestrogen derivatives and for foreign compounds such as menadione (Chasseaud, 1974). A GST-conjugate for dopaquinone has also been identified in human malignant melanoma (Agrup et al, 1977), which suggests this is also a GST substrate.

Leukotriene A_4 is converted to leukotriene C_4 by GST mediated GSH conjugation (Bach et al, 1984). The latter compound plays an important role in the mediation of local inflammatory reactions. In rats the Yb subunits appears to

catalyse this reaction, with both mu and alpha GST's in humans involved in the conversion of leukotriene A₄ methyl ester to the leukotriene C₄ methyl ester derivative (Mannervik *et al.*, 1984). This illustrates perhaps one of the important known physiological roles for the GST's. Another important physiological role of the GST's may be to protect membranes from peroxide generated lipid peroxidation (Ketterer *et al.*, 1982; Tan *et al.*, 1984). It is worthy of note that cholesterol α -epoxide (5 α ,6 α -epoxy-cholestan-3 β -ol), which is a product of lipid peroxidation, has been identified in the rat liver as a GST substrate, especially for the alpha class Ya subunit (Meyer & Ketterer, 1982), and also aldehyde products of lipid peroxidation, such as 4-hydroxy alkenals, will form GSH-conjugates (Esterbauer, 1982). In recent studies, Alin *et al.* (1985) showed that a major product of peroxidative degradation of polyunsaturated fatty acids, 4-hydroxynon-2-enal, was a good substrate for cytosolic rat GST's; the most active subunit being the Yb₂ subunit. Although Yb₁ has modest activity towards 4-hydroxyalkenals, its activity towards the highly mutagenic compound 4-hydroxypent-2-enal was found to be high compared with other isoenzymes (Marnett *et al.*, 1985). This is also interesting as the Yb₁ subunit appears to bind DNA and has been purified from rat liver chromatin (Bennett *et al.*, 1986). This polypeptide may, therefore, play a role in the protection of DNA against peroxidative damage. Human mu class (μ) and rat alpha class (Yc, Yk) GST's were also found to have high activity towards 4-hydroxyalkenals of different chain lengths (Jensson *et al.*, 1986; Danielson *et al.*, 1987), with rat subunit Ya in particular having activity towards 4-hydroxydodec-2-enal, the most toxic of these compounds (Marnett *et al.*, 1985).

Apart from their involvement in lipid peroxidation, non-selenium dependent glutathione peroxidase were also shown to reduce peroxidised DNA and were identified as GST's (Ketterer *et al.*, 1987). They differ from the selenium-dependent enzyme in that they have low activity towards organic hydroperoxides and none at all towards hydrogen peroxide (see also Section 1.9.0). The difference

in specific activity, however, is more than compensated by the quantity of the enzyme present, being 0.1-0.2mM in hepatic cytosol (Ketterer et al, 1986). Cumene hydroperoxide activity is associated mainly with the alpha class (Ya, Yc) subunits, although mu class Yb₂ GST also has activity towards this substrate (see Table 3). GST rat mu class subunits and human mu class (μ) protein were thought to be the most effective in removing peroxidised DNA (Ketterer et al, 1987), but the question of accessibility to peroxidised DNA is still unanswered.

(b) Exogenous substrates (xenobiotics)

The reaction of GST's towards xenobiotics can be further subdivided into those involving (i) detoxification; for example, alkylating agents (see Section 1.11.0), peroxides and other electrophilic compounds and (ii) toxification, i.e. the conversion of a compound to more toxic products as a consequence of conjugation with the GST's.

(i) Detoxification reactions

GST's also carry out protective detoxification reactions by catalysing the conjugation of a wide spectrum of xenobiotics including anticancer drugs to GSH (Jakoby, 1978; Chasseaud, 1979). Other electrophiles conjugated involve exogenous epoxides (Jering & Bend, 1977; Hernandez & Bend, 1982) and also a wide list of other foreign compounds (for a review, see Chasseaud, 1979). These compounds include, for example, halogenonitrobenzenes, the herbicide 2-chloro-S-triazine, aryl nitrocompounds, aralkyl halides, and alkyl halides such as bromobenze etc. The carcinogenic compound aflatoxin B₁ is also thought to be detoxified by the GST's (Manson, Personal Communication), as is the drug paracetamol (Mitchel, et al, 1973; Jones et al, 1978).

(ii). Toxification reactions

GST mediated reactions can also activate compounds. In particular, the conjugation of GSH to compounds such as hexachloro-1,3-butadiene (Wolf et al, 1984) has been illustrated in this regard. The formation of the GSH conjugate of

this compound is catalysed by GST's in the liver. It is transported from the liver to the kidney, where it is acted upon by a variety of enzymes involved in mercapturic acid formation, and, ultimately, by the cysteine conjugate β lyase enzyme. The resulting product is a reactive sulphenium ion which acts as an alkylating agent and binds to DNA inducing cytotoxic damage and tumour formation.

The substrate specificity and biological functions of the GST's, as described above, will, in conjunction with the distribution and quantity of any particular subunit, interact to influence the response of a cell to a particular cytotoxic compound.

1.10.7 Modulation and regulation of GST expression.

The regulation of GST isoenzymes within both normal and tumour cells is likely to be an important factor in determining their susceptibility to toxins and carcinogens (Wolf ^{et al} 1987a; 1987c). Cellular levels of GST's have been shown to be determined at both the genetic level and at the level of transcription. In particular, the genetic element is due to the polymorphic nature of the GST's, while the modulation of transcription element occurs through the action of both endogenous and exogenous factors.

a) Polymorphism

Genetic studies of the human GST's indicated that the GST enzymes were products of three autosomal loci GST₁, GST₂, and GST₃ (Board, 1981a; 1981b) which correspond to mu, alpha and pi GST proteins respectively. It is, at present, unclear how many gene products there are, but Board (1981b) has reported that the alpha GST's were polymorphic in man. It is thought, however, that this finding could be explained by the increased expression of one alpha subunit (B₁) over another (B₂), forming homo and hetero dimers (B₁B₁, B₁B₂) of the major protein only (Stockman et al, 1985; 1987). It is noteworthy that significant individual differences in the GST isoenzyme pattern are found in human subjects; that is, not all livers contain the same alpha class GST proteins or have detectable pi class GST

or mu class GST levels (Hayes et al, 1987c). In particular, one of the mu class (μ) GST subunits has been shown to be polymorphic in man and is absent in approximately 40% of the caucasian population (Board, 1981a; Warholm et al, 1983; Hussey et al, 1986). This enzyme has the highest specific activity towards benzo(a)pyrene-4,5-oxide, a mutagenic arene oxide of a polycyclic aromatic hydrocarbon (Warholm et al 1983; Ketterer et al, 1983). If the enzyme is nulled, it could have both toxicological and physiological significance. These differences may, therefore, have a bearing on the effectiveness of detoxification by GST's, and the subsequent response of an organ or cell to cytotoxins and carcinogens, and hence the susceptibility to cancer. In this regard, Seidegard et al, (1986) has shown that smokers who he phenotyped to have low or no mu class (μ) GST were more susceptible to lung cancer.

b) GST regulation

(i) Endogenous factors

Endogenous factors can play an important role in modulating GST levels. For example, the lymphokine interferon showed preferential induction of the Yf subunit in mouse liver and suppression of Ya, Yb, and Yc subunits (Adams et al, 1987), and there are also many studies which indicate that hormones will regulate these isoenzymes. In this respect, the pi class Yf subunit is expressed in a sex specific manner in male mouse liver (Hatayama et al, 1986; McLellan & Hayes, 1987). This effect is thought to be mediated by testosterone (Hatayama et al, 1986). In contrast, the alpha class Ya subunit expression was found to be higher in female rat and mouse livers (Hales & Neims, 1976; Igarashi et al, 1985; McLellan & Hayes, 1987). Evidence of Ya subunit regulation by hormones was substantiated by the finding that hypophysectomy or thyroidectomy led to a two fold induction of this subunit. Thyroxine would however, reverse this induction in both cases (Reyes et al, 1971; Hales & Neims, 1976).

Trace elements such as selenium are also known to influence GST isoenzyme levels. Selenium deficiency was found to cause an induction of the rat Ya, Yc, and Yb subunits (Arthur et al, 1987). Recent evidence also indicates that GST subunits can be phosphorylated in vitro, which could represent a further regulatory mechanism (Pyerin et al, 1987).

(ii). Exogenous factors

A wide variety of exogenous compounds, including those such as phenobarbital (Arias et al, 1976), 3-methylcholanthrene (Reyes et al, 1971) and trans-stilbene oxide (Guthenberg et al, 1981) are known to be inducers of cytosolic GST's, in particular in rat liver. The induction pattern varies for different subunits, indicating differential regulation of GST isoenzymes (Pickett et al, 1987). The rat liver Ya and Yb subunits tend to be those induced most significantly both at the protein (Hayes et al, 1979; Ohmi et al, 1981) and mRNA levels (Pickett et al, 1981; 1982a; 1982b). Examples of species specific induction also exist. For instance, in humans treated by phenobarbital, no increased bromosulphothalein conjugating activity was observed. Further to this, it has been found that mice are more responsive than rats, with increased bromosulphothalein conjugation in vivo, following phenobarbital administration.

Other P450 inducers, such as β -naphthoflavone, 2-aminofluorene and α -naphthoflavone, have been shown to selectively induce the Ya, Yc, and Yb subunits in rat liver (Dolan et al, 1988). In the same study, dexamethasone and β -naphthoflavone, suppressed and induced respectively, the expression of the Yf polypeptide. This is the first report of the regulation of this subunit by an exogenous organic molecule. In addition, the Ya and Yb subunit is markedly induced in rats and mice respectively, by the ingestion of the phenolic antioxidants such as 2,(3)-ter-butyl-4-hydroxyanisole (BHA) and 2,6-ditertiarybutyl-p-cresol (BHT) (Pearson et al, 1983; Patridge et al, 1983). The antioxidant ethoxyquin has also been shown to increase GST activity (Benson et al, 1978). In particular, the alpha

class GST's were elevated, and shown to be involved in the protection of rat liver towards AFB₁ induced hepatocarcinogenesis (Manson, Personal Communication). It is still unclear, however, whether the mouse Ya subunit can be induced by xenobiotics. GST induction is not confined only to mammalian species. As in corn, a GST is induced by chemicals used as antidotes to certain herbicides. The isoenzyme regulated in this manner is undetectable in untreated corn cells (Mozer et al, 1983).

More work is required, however, on the study of the regulation and modulation of GST isoenzymes, both at the protein and molecular level, and, in particular, in extrahepatic tissues. There are already described, however, complex regulatory systems involved in GST expression. Hormonal regulation, for instance, may be important in certain types of hormonal induced/dependent cancers. Xenobiotics and trace elements, such as selenium, can also influence GST levels, and, therefore, there is a possibility that these factors may influence the response of cells to carcinogens and anticancer drugs.

1.10.8 Microsomal GST.

Microsomal GST, in contrast to the cytosolic GST's, is a trimeric protein, with an apparent subunit molecular weight of 17,000. This protein was detected in rat, mouse, and human livers (Morgenstern et al, 1987; Hayes et al, 1987a, 1987c; McLellan & Hayes, 1987) by both Western blot and substrate analysis on microsomes prepared from these tissues. The function of this unusual enzyme is thought to involve the protection of membrane associated proteins and protein biosynthesis in the ribosomes from xenobiotics. The involvement of this protein in drug resistance has not been investigated.

1.11.0 **Interaction of GSH and glutathione-dependent enzymes with anticancer drugs.**

GSH and glutathione-dependent enzymes can influence the therapeutic efficacy of anticancer drugs in a variety of ways, which include modulation of detoxification, toxification, delivery, or the action of the drugs concerned

1.11.1 Detoxification

(a) Nitrogen Mustards: Mechlorethamine. Melphalan. Chlorambucil.

The ability of alkylating agents such as the nitrogen mustards mechlorethamine, melphalan and chlorambucil to react with a variety of cellular nucleophiles, including sulphhydryl groups, has long been recognised (Hirono, 1961; Connors, 1966). For example, there is evidence which indicates that increased cellular GSH levels can protect against the toxic effects of melphalan. Calcutt & Connors (1963) demonstrated a relationship between tumour growth inhibition by melphalan, an isomer of melphalan, and non protein sulphhydryl content (NPSH), in six murine tumours. Suzukake et al (1982) have also shown that a L1210 leukaemia cell line made resistant to melphalan contained a two fold higher concentration of GSH. The resensitisation of these cells, by lowering the GSH content, indicates a possible role for GSH in melphalan detoxification. Suzukake et al (1983) further demonstrated that the resistance to melphalan correlated with both GSH content and enhanced conversion of melphalan to an inactive metabolite. This conjugation of GSH to melphalan has been shown to be catalysed by glutathione S-transferases (Dulik et al, 1986), although the isoenzyme involved was not demonstrated.

(b) Oxazaphosphorines: cyclophosphamide.

Cyclophosphamide (CP) is a widely used nitrogen mustard which undergoes activation by cytochrome P-450's. The generally accepted scheme for the metabolism of this compound (Friedman et al, 1979) involves oxidation to 4-

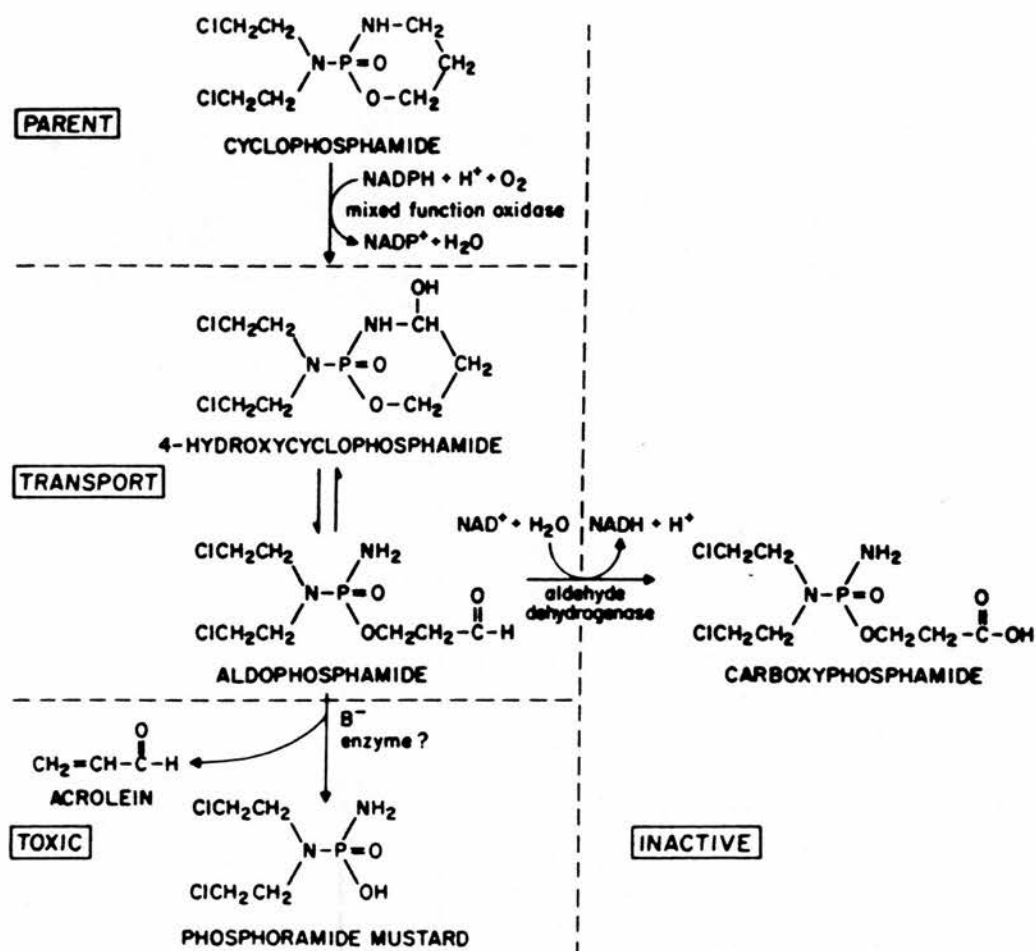
hydroxycyclophosphamide (4-OH-CP), which is in tautomeric equilibrium with aldophosphamide (Figure 7). Non-enzymatic cleavage of aldophosphamide results in the formation of two cytotoxic species, phosphoramidate mustard, the therapeutically active species, and acrolein, which is responsible for the urotoxicity of this compound (Cox, 1979). Involvement of GSH, however, in the protection from the metabolism-dependent urotoxicity of CP is well documented (Gurtoo et al, 1981), and is shown to occur without interfering with the chemotherapeutic activity of the antitumour drug (Berrigan et al, 1982). Deactivation of the 4-OH-CP metabolite of CP with GSH has been reported (Draeger et al, 1976), a reaction which would reduce the alkylating potential of this anticancer drug. Depletion of GSH levels, as carried out by Crook et al (1986a; 1986b), indicated that this protection could be reversed, and ,consequently, cause potentiation of the cytotoxicity of the activated CP compound, 4-OH-CP.

(c) Platinum compounds: cis platinum.

Cis platinum is thought to act therapeutically by cross linking DNA and inhibition of essential proteins. There has been however, a considerable interest in the role of GSH on both the nephrotoxicity and the anti-tumour effects of this compound. Cis platinum reacts with both cysteine and GSH in a second order reaction. Reaction half times are approximately 1 and 3 hours respectively, at a concentration of 5mM and 37°C. The reaction between GSH and aquated cis platinum, however, is essentially instantaneous (Dedon & Borch, 1987). This suggests that direct participation of GSH in reducing cytotoxicity occurs via reaction with metabolites, rather than with cis platinum itself.

Depletion of renal GSH levels by BSO increases cis platinum lethality, while pretreatment with GSH causes a dose-dependent reduction in its lethal effects. Hamilton et al (1985) has also shown that depletion of GSH levels in a cis platinum resistant human ovarian carcinoma cell line (A2780) (exhibiting a 3 fold increase in GSH levels over the wild type line), increased the cytotoxicity to cis platinum.

Figure 7. Metabolism of cyclophosphamide: an abridged scheme showing only the currently recognised biologically important reactions and metabolites.
(Taken from Sladek, 1987).



Elevated GSH may not be a prerequisite for cis platinum resistance, as Andrews *et al* (1985) did not find any change in GSH levels in the cis platinum resistant cell line. In addition, the cells were not resensitised to cis platinum on addition of BSO. There are, however, three possible explanations for the different responses to glutathione depletion in these two groups. First, the removal of BSO during cis platinum treatment by Andrew *et al* (1985) may have allowed GSH levels to recover sufficiently to negate the effect of prior depletion. Second, depletion of GSH may not have been low enough to allow potentiation of cis platinum cytotoxicity, and, thirdly, other mechanisms of resistance may have existed in those cells to allow further protection, such as elevated DNA repair capacity. In addition, the capacity of cells to increase GST activity may be important, for example, Teicher *et al* (1987) has shown that in a cis platinum resistant cell line no difference in GSH levels was measured, but an increase in GST activity was found. Whether the cell line was cross-resistant to other compounds was not shown.

d) Anthracyclines: Adriamycin.

Antineoplastic quinone-containing drugs such as Adriamycin (Adr) are thought to undergo metabolic activation by microsomal enzymes to free radicals, which can interact with oxygen to generate reactive oxygen intermediates via superoxide anion (Bachur *et al*, 1978). These drugs in the presence of iron can cause lipid peroxidation (Sugioka, 1981; Doroshaw, 1983) and DNA strand breakage (Berlin & Haseltine, 1981). Analysis of the anti-oxidant defences of mouse heart has implicated selenium-dependent GPX as a major pathway for the detoxification of reactive oxygen intermediates generated by adriamycin (Doroshaw *et al*, 1980).

e) Nitrosoureas: 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)

Nitrosoureas are a group of alkylating agents widely used against many different types of cancers. In addition, however, the nitrosourea BCNU is also

shown to inhibit glutathione reductase (GRD) (Frischer & Ahmad, 1977; see Section 1.8.0), which may have an important role not only in reducing free BCNU levels, but also influencing the response of cells to other cytotoxic agents which could be detoxified by GSH. For example, incubation of isolated rat hepatocytes with BCNU with adriamycin concomitantly, leads to potentiation of Adr cytotoxicity (Babson et al, 1981). This indicates the involvement of the redox cycle in the mechanism of Adr induced cytotoxicity, and illustrates the importance of maintaining the thiol status of the cell. Interestingly, there is also preliminary evidence to suggest the conjugation of GSH to BCNU can be catalysed by GST's in the brain, and, in particular, the mu class protein is thought to be involved (Smith et al, 1987).

f) 6-Thiopurine.

6-Thiopurine is metabolised to its active form by cytochrome P-450's. This reactive metabolite binds irreversibly through a disulphide linkage to proteins both in vivo and in vitro (Hyslop & Jardine, 1981a; 1981b). This binding is decreased in the presence of GSH, and GSSG is formed. The proposed mechanism of protection is thought to involve GSH competing with protein sulphydryl groups with the drug metabolite purine-6-sulferic acid, forming thiopurine GSH mixed disulphide. This metabolite reacts with another GSH molecule to regenerate 6-Thiopurine and GSSG.

6-Thiopurines can also be metabolised by the purine de novo biosynthesis pathway. In this situation, 6-Thiopurines act as antimetabolite anticancer drugs. The involvement of GSH and glutathione-dependent enzymes in this metabolic pathway of 6-Thiopurines has not been studied.

1.11.2 Toxification.

a) Azathiopurine.

Azathiopurine is the precursor form of 6-Thiopurine, which is converted into 6-Thiopurine and various methyl nitromidazole metabolites by thiolysis, involving GSH (De Miranda et al, 1973).

b) Bleomycin.

Bleomycin is a mixture of two structurally similar glycopeptides, which is thought to be cytotoxic by an oxygen-dependent mechanism of DNA cleavage (Onishi et al, 1975; Sausville et al, 1978a; 1978b). Specifically, a ferrous iron-bleomycin complex reduces molecular oxygen to generate a superoxide anion while oxidising the iron atom. Caspray et al, (1981) demonstrated that GSH is able to bind to the ferric iron/bleomycin complex and reduce it to the active ferrous form. Similar oxidative-reductive metabolism of other anticancer drugs, such as neocarzinostatin, have been proposed, with GSH playing a vital role (De Graff et al, 1985).

1.11.3 Delivery/Transport.

Methotrexate.

GSH can affect the rate of uptake of anticancer drugs. For example, the uptake of Methotrexate (MTX) is increased in rat hepatocytes on the addition of GSH to the medium (Leszczynska & Pfaff, 1982). This effect may be mediated by effects on membrane sulphydryl groups, which are implicated in this process (Leszczynska & Pfaff, 1982). Inhibition of uptake was seen if the cells were exposed to the sulphydryl oxidising agent N-methyl diazenedi-carboxylic acid bis (N-methyl piperazide). Addition of GSH reversed this inhibition, probably due to reactivation of critical sulphydryl groups (Leszczynska & Pfaff, 1982).

The above discussion provides evidence that alterations in GSH content and certain glutathione-dependent enzymes can affect the detoxification, toxification,

transport, and effectiveness of a variety of anticancer agents. The enzymes which maintain GSH levels will also, therefore, play a central role in these effects.

1.12.0 Drug Resistance.

In the context of this thesis the word drug is used in general terms, and is defined as a toxic chemical, which also includes foreign compounds.

The development of drug resistance in tumours to anticancer drugs has been recognised as one of the major obstacles to successful chemotherapy for a wide variety of tumours. There are several reasons which will explain this effect, which include: a) tumour growth: a low growth fraction of a tumour may be refractory to treatment, as most anticancer drugs are proliferation-dependent in their action; b) in vivo distribution of drug: sanctuary sites for the drug may exist, such as the central nervous system and testis, where metastatic tumours do not get exposed to the drug ; c) drug diffusion limitations: there are several classes of anticancer drugs, such as the anthracyclines, which are hydrophilic in nature and unable to penetrate into poorly vascularised and necrotic tumours; d) hypoxia effects: poor vascularisation of the tumours will also affect both the bioavailability of the drug and oxygenation, which is important for the therapeutic action of certain anticancer drugs, such as bleomycin, and e) biochemical effects: changes in the biochemical profile of the tumour may affect the response of the tumour to the anticancer drug used. The possible biochemical mechanisms of drug resistance include altered drug uptake and efflux (Kartner et al, 1983), DNA repair (Karran & Lindahl, 1985), gene amplification (Stark & Wahl, 1984) and drug detoxification involving glutathione-dependent enzymes as well as others (Chasseaud, 1979; Larsson et al, 1983a; Wolf et al, 1987a; 1987c; Hayes & Wolf, 1988).

In view of its potential importance, recent studies on drug accumulation are worthy of note. A protein has been identified in cells resistant to a wide variety of structurally diverse cytotoxic natural products, such as colchicine, vinca

alkaloids, actinomycin D and anthracyclines (multidrug resistance, MDR) (for review see Beck, 1987; Gottesman & Pastan, 1988; Moscow & Cowan, 1988). This protein is glycosylated, and termed GP 180 or P-glycoprotein (Juliano & Ling, 1976). The evidence that GP 180 mediates MDR resulted from studies which showed that GP 180 was over expressed in cell lines (Bech-Hanson et al, 1976; Harker & Sikic, 1985; Cornwell et al, 1986; Gros et al, 1986). GP 180 has also been identified in both normal and tumour tissues (Fojo et al, 1987).

It is important to note that mechanisms of resistance are not mutually exclusive, and more than one mechanism may occur in any particular cell, or population of cells, within a tumour. Other models of drug resistance have also been described. These include the resistance of normal cells to a normally lethal dose of chemotherapeutic agent, following a low protective dose (Miller et al 1975; Boyd, 1980; Adams et al, 1985; Carmichael et al, 1986); the resistance of liver cells to carcinogens (Farber, 1984a); resistance of cells to oxidative challenge (Kimball et al, 1976; Sedgwick & Robins, 1980; Christman et al, 1985; Morgan et al, 1986; Shelton et al, 1986); the resistance of cells to viral infections, i.e. action of interferons (for a review see Revel & Chebath, 1986) and heat shock (for a review see Lindquist, 1986). Whether these mechanisms of resistance involve similar controlling factors has not yet been clearly established.

1.13.0 **Adaptive Response.**

It is, as yet, unclear whether tumour cell acquired drug resistance is related to an adaptive response as a consequence of administration of the anticancer drug, or to a selection for intrinsically resistant cells. The former case results from an elevation of enzymes which protect against the cytotoxic effect of the drug. It has been shown by Christman et al (1985), that GSH, and certain glutathione-dependent enzymes, can be elevated following oxidative stress in bacteria, which supports the possibility of an adaptive response. A similar response has also been shown in mammalian cells (Sedgwick & Robins, 1980). The

inducible repair pathway to counteract the effects of alkylating agents in bacteria (Samson & Cairns, 1977; recent review, Lindahl, 1987) also provides evidence to suggest that an adaptive response to alkylating agents involving the induction of a series of enzymes in mammalian cells is not unreasonable.

1.14.0 **Objectives of this study.**

The major aim of this project is to study whether there is a common underlying mechanism of drug resistance involving GSH and glutathione-dependent enzymes, especially in the acquired drug resistance of tumour cells to cytotoxic drugs. The extent to which such changes represent a common response will be studied by investigating other drug resistant models. On the basis of previous work carried out in the laboratory by Adams et al. (1985) and Carmichael et al. (1986), there is evidence to suggest that GSH and glutathione-dependent enzymes may be involved in an adaptive response to cytotoxic compounds. In particular, it was shown that GSH and GST activity was elevated in normal bone marrow cells in response to a low dose of cyclophosphamide, a chemotherapeutic agent, which afforded protection against a higher lethal dose. One of the objectives of this investigation is to look at this resistance phenomenon in more detail, to identify which GST subunits are involved, and whether other glutathione-dependent enzymes are also elevated. The other drug resistant models which will be investigated include the response of liver cells to carcinogens, and the response of lung cells following exposure to high oxygen concentrations. Whether the mechanism of acquired drug resistance in tumour cells is due to an adaptive response as a consequence of the administration of a cytotoxic drug, or to a selection of intrinsic resistant cells, will also be determined.

All chemicals were purchased from commercial sources and were of the highest grade of purity available. Unless otherwise indicated, the chemicals were obtained from BDH, Poole, Dorset.

2.1.0 Cell Culture

2.1.1 Growing and routine maintenance of cells.

All cell lines were taken from laboratory stocks except the Chinese hamster cell lines CHO-K1/CHO-Chl^r and the human sarcoma cell lines MES-SA and DX5 which were obtained from Mr. C. Robson and Dr. I. Hickson, Cancer Research Unit, Royal Victoria Infirmary, Newcastle-upon-Tyne and Professor B.I. Sikic, Stanford University, California, respectively. Human breast carcinoma MCF7, ovarian adenocarcinoma PE01/PE04; bladder carcinoma, EJ; lung carcinoma H322/H358; foetal lung fibroblast EF484 and colonic carcinoma HT29 were grown in RPMI 1640 media (GIBCO Ltd., Paisley, Scotland). LS174T, a human colonic carcinoma line was grown in minimum essential medium (MEM) containing non essential amino acids (GIBCO Ltd, Paisley, Scotland) and human hepatoma lines HEPG2 in Dulbecco modified eagles medium (DMEM) (Northumbrian Biologicals Ltd, Newcastle). The two CHO cell lines CHO-K1 and Chl^r were maintained in Hams F10, and the human sarcoma lines MES-SA and DX-5 and mouse hepatoma, Hepa 1 in Hams F12. Both media were obtained from GIBCO Ltd., Paisley. All the media were supplemented with 10% (v/v) foetal calf serum (FCS) (GIBCO Ltd., Paisley, Scotland), streptomycin (100µg/ml) and penicillin (100 IU/ml) (GIBCO Ltd., Paisley, Scotland). Insulin (2.5 mg/ml) SIGMA, Poole, Dorset) was also added to cultures of PE01 and PE04. Cell lines were cultured at 37° C, 100% humidity and 5% CO₂. When cells were not immediately used, they were cryopreserved in freezing mixture of 90% newborn calf serum (GIBCO Ltd., Paisley, Scotland) and 10% dimethyl sulphoxide (SIGMA, Poole, Dorset) at 50 to 100 x 10⁶ cells/ml. Controlled

cooling to -80°C was carried out before storage in liquid nitrogen. When used, the cells were rapidly brought to 37°C and washed twice in the appropriate medium to remove the dimethyl sulphoxide. Cells were seeded in a $1 \times 25\text{cm}^3$ flask (GIBCO Ltd., Paisley, Scotland) and left overnight to settle before refeeding. Cultures of cells were refed usually three times a week, and subcultured as necessary. Subculturing (passage) involved harvesting the cells with 0.1% (w:v) trypsin (Difco Labs., West Molesley, Surrey) and 0.001% (w:v) versene (EDTA) 1:1 mix, and splitting the cell suspension, in order to leave the cells in logarithmic growth. Cells were used experimentally only up to 5 passages, after this period they were discarded and fresh cryopreserved cells at an earlier passage were used. This was to avoid alteration in cell phenotype, which may occur in cells grown for prolonged periods in culture.

2.1.2 Mycoplasma testing.

Periodic assays for mycoplasma were carried out routinely by the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh and were always found to be negative. The assays involved the fluorescence test and also the growth of mycoplasma on defined agar plates.

2.1.3 Harvesting, viability and counting of cells.

Cultures of cells in either $1 \times 75\text{cm}^3$ or $1 \times 175\text{cm}^3$ flasks (GIBCO, Paisley, Scotland) were harvested with 0.1% (w:v) trypsin and 0.001% (w:v) versene, 1:1 mix, washed three times in phosphate buffered saline (140MM NaCL, 2.7 mM KCl, 8MM sodium phosphate pH 7.4) (Oxoid, Basingstoke, Hampshire) and resuspended in 10mls of phosphate buffered saline (PBS) for counting and viability. Determination of cell number was assessed by using a hemocytometer. Cell viability was determined by mixing 100 μl of cell suspension with 100 μl of 0.5% (w:v) nigrosin (Sigma, Poole, Dorset) and looking for viable cells under phase contrast microscopy. Viable cells do not take up nigrosin dye and therefore appear bright, in contrast to dead cells

which appear dark in colour: from this the viability of the cell preparation was assessed.

2.1.4 Synchronisation of cells.

Flasks were seeded at 2×10^5 or 5×10^4 cells/ml to obtain either confluent cells or cells in logarithmic growth respectively. Cell synchronisation was achieved by arresting the cells in Go/G1 of the cell cycle by reducing the concentration of foetal calf serum in the medium from 10% (v:v) to 0.5% and leaving the cells for 6 days (Balkwill and Bokhonik, 1984). Synchronised logarithmic growth was obtained by increasing the FCS concentration back to 10%. Cells were then harvested 18 hours later as described in Section 2.1.3.

2.1.5 Thymidine incorporation.

In order to confirm logarithmic growth phase, thymidine incorporation into the DNA was measured using radiolabelled thymidine (Heyns *et al*, 1985). Following incubation of the cells with medium containing 0.19 Mbq ^3H methyl thymidine for 4 hours, the cells were washed with PBS, harvested and 50 μl aliquots of the cell suspension (1×10^7 cells/ml) absorbed onto Millipore filters. Samples were then washed sequentially with distilled water, 5% (w:v) trichloroacetic acid, distilled water and methanol. Filters were oven dried, 1ml of unisolve (Sigma, Poole, Dorset) scintillation fluid added, left for 1 hour and radioactivity determined by scintillation counting.

2.2.0 **Cell Preparation for Biochemical Assays.**

Cell cultures were harvested with 0.1% (w:v) trypsin and 0.001% versene (EDTA) , 1:1 mix, washed three times in PBS and resuspended in 400 μl of PBS. Cell viability was determined to be greater than 95% as assessed by nigrosin dye exclusion. The cell suspension was lysed by sonication with three 5 second pulses at maximal power (MSE Soniprep 150). A 5 second cooling period at 4°C was included between each burst. The



resulting sonicate was spun at 18,000g average for 20 minutes (Eppendorf 5414) and the supernatant decanted. The supernatant (soluble fraction) and pellets were collected and stored at -70°C until required.

2.3.0 **Drug Resistance in Animal Models.**

2.3.1. Chemotherapeutic drug induced resistance in normal bone marrow cells.

Male CBA mice (23 to 28g) were obtained commercially from Bantin and Kingman. They were allowed food and water ad libitum and kept in a controlled environment with 12 hours light. Treated ('primed') animals received cyclophosphamide (Sigma, Poole, Dorset) as a non lethal 'priming' dose of 75mg/kg injected intra-peritoneally (i.p.) in a solution of 0.1ml 0.9% (w:v) sterile NaCl solution (Saline) per 10g body weight. Control mice received just saline injected i.p. Animals were killed by cervical dislocation on days 5 and 6 after i.p. injection.

(a) Preparation of bone marrow cells.

Bone marrow samples were obtained from femurs from 3 animals, Buffer A (1.5ml) containing 20mM KP04, 2.3% KCl, 0.2mM EDTA pH 7.7 was flushed through the severed femurs. Bone marrow cells were pelleted at 18,000g average for 5 seconds (Eppendorf 5414) and resuspended in 1ml of buffer A. The cell concentrations in the bone marrow samples were determined using a haemocytometer. These samples were respun and resuspended in 400µl of KCl-phosphate (10mM KP04, 1.15% KCl, 0.1mM EDTA pH 7.7), sonicated with three 5 second pulses at maximal power to lyse the cells, with a 5 second cooling period at 4°C included between each burst. The resulting sonicate was spun at 18,000 g average for 20 minutes and the supernatant decanted. The supernatant (soluble fraction) and pellets were collected and stored at -70°C until required.

(b) Preparation of peripheral erythrocytes.

Peripheral blood samples were obtained from treated 'primed' and control 'unprimed' mice by 'eye bleeding' using a heparinised capillary tube. Blood was dropped into isotonic saline and spun in at 650 g average for 5 minutes at 4°C (Sorvall RT 6000). The serum and 'buffy coat' containing the white cells were removed and the erythrocytes resuspended in 1ml of KCl-phosphate for cell determination as described for the bone marrow preparations. The soluble fraction was obtained as described previously for the bone marrow cells.

(c) Preparation of peripheral granulocytes.

Peripheral granulocytes were prepared by inducing granulocyte accumulation in the peritoneum of mice by i.p. injections of 2ml of 0.2% (w:v) calcium/sodium caseinate (Sigma, Poole, Dorset) (Watt *et al.*, 1979) in isotonic saline. After 3 hours the animals were killed by cervical dislocation and 5 ml of isotonic saline injected i.p. The peritoneal exudate was withdrawn after gentle peritoneal massage, and the cells were spun down at 650 g average for 5 minutes at 4°C. Pellet was washed three times with ice cold 0.168M NH₄Cl, and left to stand for 10 minutes to lyse erythrocytes. Cell concentration and preparation of soluble fraction were carried out as described for the bone marrow cells. Purity of the sample was assessed using Giemsa staining. A slide smear from a drop of the cells was air dried and the sample fixed with a 3:1 (v:v) of methanol and acetic acid. Slides were rinsed with buffered distilled water, pH 7.0, and stained for 15 minutes with Giemsa stain diluted 1:10 (v:v) with buffered distilled water, pH 7.0, (stock Giemsa was prepared by adding 4g of Giemsa powder [Sigma, Poole, Dorset] to 250 mls of glycerol heated to 60°C, until dissolved. 250mls of methanol was then added and the solution was stirred and left to stand for 7 days before filtering). Slides were

rinsed with ^{phosphate} buffered distilled water, pH 7.0, and allowed to dry before assessing purity of the preparation by phase contrast microscopy. The peritoneal granulocyte fraction of cells was determined to consist mainly of granulocytes.

(d) Preparation of peripheral lymphocytes.

Spleens were rapidly removed from 'primed' and control mice and washed in buffer A, before scissor mincing in a small amount of isotonic saline. This was subsequently homogenised using a teflon/glass hand held homogeniser and the homogenate filtered through a tea strainer. The resulting preparation was spun at 650g average for 5 minutes in a sorvall RT 6000 refrigerated centrifuge and the pellet resuspended in 4.5 mls of distilled water, and left for 12 seconds to lyse the erythrocytes. Immediately, 0.5ml of 10X concentrated PBS was added to the tube and was topped up with isotonic PBS. This was respun at 650g average for 5 minutes and the last two steps repeated if necessary, to remove any erythrocyte contamination. The resulting lymphocyte pellet fraction was resuspended in 1ml of KCl-phosphate, and the cell count determined as described for the bone marrow preparations. Giemsa staining for purity was as mentioned for the granulocyte preparation, and was found to contain mainly lymphocytes. The soluble fraction was obtained as described for the bone marrow preparations.

(e) Fluorescence activated cell sorting of bone marrow cell types.

The fluorescence activated cell sorting of the bone marrow cell types was carried out in collaboration with Dr. J. Ansell, Dept. Zoology, University of Edinburgh.

For cell sorter (FACS IV; Becton Dickinson) analysis and sorting, bone marrow cells were obtained by flushing femurs with 1ml of cold buffered isotonic saline supplemented with 0.1% bovine serum albumenⁱ and 0.02% EDTA. The various cell types from the bone marrow were separated on the

FACS on the basis of the differential forward and right angle scattering properties (Watt et al 1980; Ritclue et al 1983). The 488-nm line of an argon ion laser was used for the excitation of both forward and right angle scatter signals, and the samples were run at approximately 1,000`cells/second. Appropriate gates were set around granulocytes, lymphocytes and a third mixed cell population (mainly monocytes) from the bone marrow preparations, and these populations were sorted into cooled eppendorf tubes (Figure 8).

Approximately 300,000 cells were obtained for the granulocyte and lymphocyte populations. The soluble fractions were prepared as described for the bone marrow cells except the cells were resuspended in 100µl of KCl - phosphate.

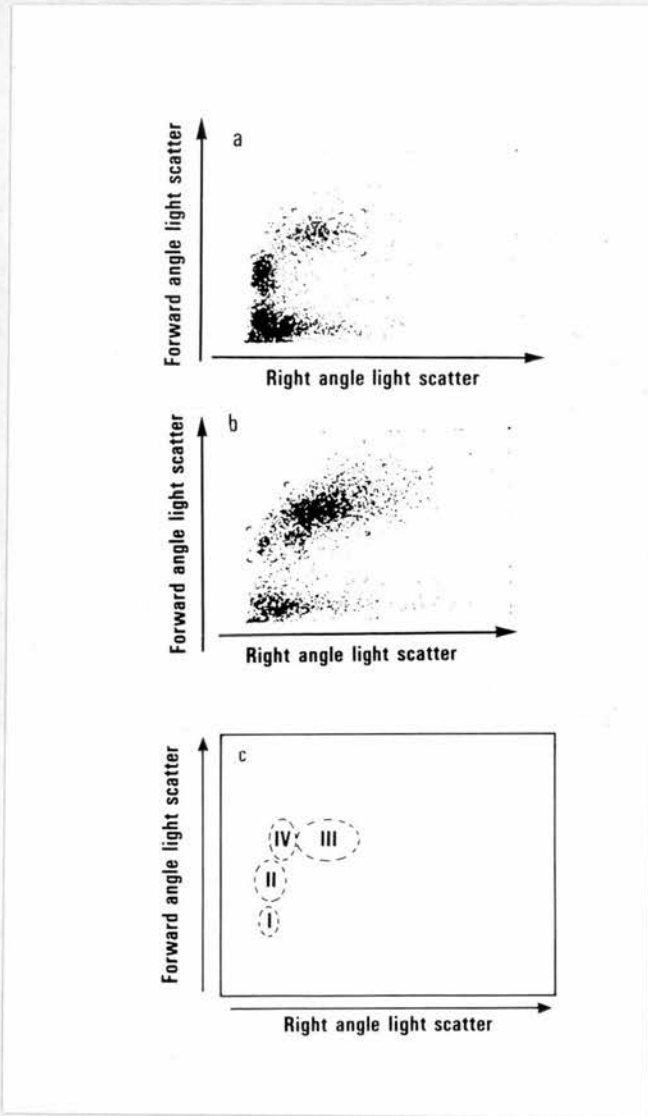
2.3.2 Resistance of normal lung cells to oxygen.

The generation of the oxygen resistant rats was carried out in collaboration with Mr. I. Wyatt, Biochemical Toxicology Section, ICI Plc, Macclesfield, Cheshire.

Male, specific pathogen-free ALPK:AP (Wistar-derived) albino rats (175-200g) were supplied from the colony maintained at Alderley Park, Cheshire. They were allowed food, pellet Porton Combined (PC) diet, supplied by Special Diet Services Ltd., Witham, Essex, and water ad libitum. Conditions were kept at a 12 hour light/dark cycle with temperature and humidity at 21-22°C and 40-60% respectively. Five treated rats were exposed to oxygen (Air Products Ltd) from cylinders mixed with air to give a concentration of 85-90% (v:v) oxygen, in a circular whole body exposure chamber (approximately 5 litres of oxygen per minute mixed with 600mls per minute of air). The oxygen concentration was monitored and recorded continuously throughout the exposure using a Beckman Field lab oxygen monitor. Control rats were housed in the holding room under normal conditions. Seven days later the rats were killed one by one with an overdose of fluorothane, the heart

FIGURE 8. FACS Dot Plots of Cells from Bone Marrow

FACS IV dot plots of mouse bone marrow from untreated animals (a) and animals treated 5 days previously with cyclophosphamide (CP) (75mg/kg) (b). The schematic scheme (c) indicates the position of the cell populations separated as described in Materials and Methods (Section 2.3.1), I, erythrocytes; II, lymphocytes; III, granulocytes; IV, mixed cell population.



and lungs dissected out, and the lungs perfused with ice cold isotonic saline containing 5mM EDTA (degassed with N₂). The lungs were removed from the heart, placed in tubes, and frozen at -70°C until used. The livers were also removed from the rats and treated similarly.

Lung tissues from the control and oxygen exposed rats were pooled to give three separate samples; the livers were left individually. The tissue samples were dried, weighed, and scissor minced in a ratio of 1:3 (w:v) tissue to KCl-phosphate. The preparations were homogenised in a tissue grinder and spun in a Sorvall RC-5B reffridgerated superspeed centrifuge at 5000g average for 20 minutes at 4°C. Supernatant was poured off quickly and the pellet discarded, the resulting supernatant fraction was respun in the Sorvall ultracentrifuge OTD 65B at 240000g average for 1 hour at 4°C. The soluble fraction, obtained as the supernatant from the ultracentrifuge spin, was kept at -70°C before being assayed.

2.3.3 Hepatic preneoplastic and neoplastic nodules.

Aflatoxin B₁ (AFB₁) induced preneoplastic and neoplastic lesions in the rat were obtained from Dr. M. Manson, MRC Toxicology Unit, Carshalton.

Male Fischer F344 rats were used, bred at MRC Toxicology, Carshalton, and were routinely maintained on MRC 41B diet ad libitum. The preneoplastic foci were induced by the injection of 0.25mg/kg aflatoxin B₁ (AFB₁) i.p., followed by 2 weeks on control diet and 17 weeks of AFB₁ administered in the diet at 1 part per million (1p/pm), using a mixture of 75% (w:w) of naturally contaminated ground nut meal, 25% (w:w) powdered MRC 41B containing 2% (v:w) arachis oil. The neoplastic foci were induced in a similar manner but only had AFB₁ in the diet for 14 weeks (1p/pm) and then left on control diet for 1 year. Two different foci, Tumour A and B were obtained rom the same liver using this method. Control livers were obtained from rats which were age matched to those in which preneoplastic foci were induced. The

preneoplastic and neoplastic foci were demonstrated, immunohistochemically by Dr. M. Manson, to be γ GT positive. Soluble fractions of the preneoplastic foci and neoplastic livers were made by Dr. M. Manson from 20% (w:v) homogenates of the tissue in 150mM KCl, and by centrifuging at 100,000g average for 1 hour. Samples were stored at -70°C before use.

2.4.0 Protein Estimation.

Protein content of samples were estimated by the method of Lowry et al (1951) using bovine serum albumin fraction (IV) (Sigma, Poole, Dorset) as a standard. The protein reagents used were as follows:

- A) 70mM NaCO₃ 10H₂O : 40mm NaOH
- B) 40 mM CuSO₄ 5H₂O
- C) 71mM Na K tartrate
- D) Stock Folin Coicalteau reagent diluted 1:2 (v:v) with distilled water.
- E) Alkaline copper solution which was made up fresh by adding 0.5ml of both B and C and making the volume up to 50mls with Solution A.

Soluble fraction from the cells were diluted 1:20 (v:v) with 0.1M NaOH to give a final volume of 1ml, and soluble fractions from tissues were diluted to give a value in the range of the standards. To 1ml of sample solution, 5mls of solution E was added and vortex mixed. 10 minutes later, 0.5ml of solution D was added, vortex mixed immediately and left for at least 30 minutes. The absorbance reading of the solution at 600nm was determined in glass cuvettes. The amount of protein in the sample was derived from reference to a standard curve at 600nm, using 0, 25, 50, 80, 100, 120, 150, 200 μ g/ml bovine serum albumin, generated by a programme in a Shimadzu UV 160 spectrophotometer. A new standard curve was constructed for each new set of assays. If protein

sample was very low, as was the case sometimes for the cell samples, or if sample volume was low, a microassay was employed. This involved scaling down the volume of sample and solutions used by a factor of 5.

2.5.0 SDS Polyacrylamide Gel Electrophoresis.

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970).

Glass plates (12cm x 14cm) and spacers were wiped with alcohol, assembled into a gel sandwich and put into the Biorad protean 1 apparatus (Biorad, UK Ltd). A 12% (w:v) polyacrylamide separating gel that incorporated 0.32% (w:v) bisacrylamide was prepared using 14.8 mls of 30% (w:v) acrylamide (Sigma, Poole, Dorset), 9.25mls of separating gel buffer (1.5M tris, 0.5% SDS, pH 8.8) and 11.0 mls of distilled water. The acrylamide, buffer and distilled water was mixed thoroughly. Ammonium persulphate 2mls 1% (w:v) (APS) and 20 μ l of N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, Poole, Dorset) were added, and the preparation mixed again thoroughly. The gel was then poured immediately to a height of 12cm and gently over-layered with distilled water. The gel was allowed to set for 30 minutes. If the separating gel was to be left overnight, the water over-layer was replaced with separating gel buffer diluted 1:4 (v:v) with distilled water and the gel sealed-off with clingfilm and placed at 4°C. In order to put on the stacking gel, the overlay was completely removed and a 20 or 25 track comb, which was rinsed with alcohol and dried, inserted. The 4.5% (w:v) polyacrylamide stacking gel was prepared using 1.5mls of 30% acrylamide, 2.5 mls of stacking gel buffer (0.5M tris, 0.5% SDS, pH 6.8) and 5.7 mls of distilled water. 0.3mls of 1% APS and 10 μ l TEMED was added, mixed and the preparation poured onto the gel. After 30 minutes, when the stacking gel had set, the comb was gently removed and the apparatus inverted to remove any remaining stacking gel buffer. The slots were filled with electrode buffer

(0.52M tris, 0.53 M glycine, 35mM SDS, pH 8.3) which had been diluted 1:10 (v:v) with distilled water before use and the gel attached to the upper reservoir. To the lower reservoir of the protean apparatus 1 litre of the diluted electrode buffer was added, and the gels and upper reservoir inserted. Electrode buffer was subsequently added to the upper reservoir. Slots were flushed to remove any debris and samples were loaded with a Hamilton syringe (Hamilton Bonaduz, Bonaduz, Switzerland). The samples had been mixed 1:1 (v:v) with boiling mix (10% (v:v) stacking gel buffer, 2% (w:v) SDS, 5% (v:v) β mercaptoethanol, 10% (v:v) glycerol and 0.005% (w:v) Bromophenol blue) and heated at 100°C for 5 minutes prior to loading. Lid of the apparatus containing the electrodes was placed on top, with the current run at 20mA/gel while the dye front was in the stacking gel, and 30mA/gel in the separation gel. If required, gels were stained for 1 hour with 0.25% (w:v) coomassie blue and destained in 5% (v:v) methanol 7% (v:v) acetic acid to show the protein pattern.

2.6.0 'Western' Blot Analysis.

'Western' blots were carried out using essentially the method described by Towbin et al (1979), as modified by Adams et al (1985).

SDS-PAGE was performed as described in Section 2.5.0. Following electrophoresis, the gels obtained were treated as follows. The stacking gel was removed, the separating gel cut along the dye front at the bottom right hand corner to allow correct orientation at a later period. Electroblotting buffer (20mM Na₂HP0₄, 20% (v:v) methanol) was put into the Biorad transblot apparatus (Biorad UK Ltd) and the gel orientated in the presoaked transblot cassette as follows:- cassette, scotchbrite pad, 3mm paper. 0.45 μ m nitrocellulose (Anderman Co. Ltd, U.K.), gel, 3mm paper and Scotch Brite pad, cassette. The cassette was placed in the transblot apparatus with the nitrocellulose nearest the anode, and blotted overnight at 0.25A. After

transblotting, the cassette was removed from the apparatus and placed on the bench with the nitrocellulose below the gel. Nitrocellulose was cut to the exact size of the gel, and the track boundaries marked as necessary. The gel was discarded or stained, as described in Section 2.5, to determine the efficiency of the protein transfer, and the nitrocellulose treated as follows.

Nitrocellulose filters were washed for two 10 minute periods in 50mM Tris/ HCl, pH 7.9, 0.15 M NaCl, 0.05% (v:v) Tween 20 (Sigma, Poole, Dorset) TBST, and blocked with 3% low fat milk for 1 hour. Following two further 10 minute washes with TBST, nitrocellulose filters were incubated for 1 hour with the specific antibodies raised against the GST subunits (see Section 2.7.0). The antisera was diluted 1:500 (v:v) in TBST. Four 15 minute washes with TBST were then carried out, followed by incubation for 1 hour with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Scottish Antibody Product Unit, Glasgow, Scotland) at a dilution of 1:1000 (v:v) in TBST. Antibodies were used approximately three times before discarding. Following further washes with TBST, bands were visualised using either:

(a) Peroxidase staining using 4-chloro-1-naphthol as substrate.

Filters were incubated at room temperature in 200mls of TBS containing 3.4M 4-chloro-1-naphthol (Sigma, Poole, Dorset) and 80 μ l of 30% (v:v) hydrogen peroxide until the bands became visible. The reaction was then terminated by placing the nitrocellulose filters into distilled water. Further enhancement of the bands could be achieved by subsequent labelling with 125 I protein A followed by autoradiography.

(b) Autoradiography using 125 I protein A.

Nitrocellulose filters were incubated with 50mls of TBST containing 0.19mBq 125 I protein A (Amersham International PLC, Amersham, Bucks) for 1 hour. They were then washed with TBST until counts in the washes reached background and the filters blotted dry. Filters were put onto blotting paper,

covered in clingfilm and put into an X-ray cassette with intensifying screens. A sheet of Kodak XAR 5 film (Kodak UK Ltd) was put on top, the cassette closed, and put at -80°C. Films were developed at differing time periods as necessary, either manually or using an Gevomatic 60 (Agfa-gevaert) automatic developer.

2.7.0 Antisera Raised against Glutathione S-transferase Subunits.

The antisera raised against the glutathione S-transferase (GST) subunits used in this research, were generously given by Dr. J. Hayes at the Dept. Clinical Chemistry, Edinburgh Royal Infirmary, Edinburgh.

Antisera used was raised against rat alpha class subunits Yc, Ya, Yk; mu class Yb₁, Yn and pi class Yf subunits, and also against human alpha (B₁B₁); mu (μ) and pi (λ) subunits. Purification of the rat GST subunits, raising of the antisera and the specificity of the antisera have been described by Hayes & Mantle (1986b). Hayes & Mantle (1986b) described the cross reactivity of the rat GST antisera using Western blot analysis. Both alpha (Yc) and pi (Yf) antisera was specific for these GST isoenzymes, while the alpha (Yk and Ya) and mu (Yb₁) cross reacted slightly with other GST's of a similar class, but only at high antibody concentrations. Yn antisera reacted slightly weakly with other mu class GST isoenzyme proteins (Yb₁/Yb₂).

The human GST subunits were purified and antisera raised as described by Hayes et al (1983; 1987d) and Stockman et al (1985). Relative cross reactivity of the various antisera, determined by radioimmunoassay, found no cross reactivity with the other specific GST subunits (Hayes et al 1987c). However, on 'Western' blots, both the B₁B₁ and B₂B₂ antisera cross reacted with the B₁B₁ protein (Stockman et al 1987).

2.8.0 Assays for Gutathione (GSH) Levels.

There are a number of procedures for the determination of glutathione (GSH) and/or glutathione disulphide (GSSG) in biological samples. These include chemical (Hissin & Hilf, 1976), enzymatic (Griffith, 1980) or chromatographic procedures (Reed, et al 1980; Newton et al 1981; Nakamura & Tamura, 1982; Demaster et al 1984). It was important, however, to choose an assay which was quick, easy, and sensitive enough to detect GSH levels in cell lines. Consequently, the fluorometric assay of Hissin & Hilf (1976) was used, with the accuracy of the determination of GSH levels in cell lines confirmed by a more sensitive and accurate h.p.l.c. method.

(a) Fluorometric assay using ortho-phthaldehyde.

Soluble fractions from cells or tissue samples were taken and mixed 1:1 (v:v) with 10% (w:v) trichloroacetic acid to precipitate out proteins and acidify the sample which prevents GSH auto-oxidation. Triplicate aliquots were taken and diluted to 100 μ l, and 1.8ml of 0.1M NaPO₄:5mM EDTA pH 8.0 buffer added. 100 μ l of freshly prepared ortho-phthaldehyde 1mg/ml in methanol (OPT) (Sigma, Poole, Dorset), was added to each sample tube, mixed and left in the dark at room temperature for 15 minutes. Determinations of the fluorescence intensity at excitation 350nm and emission 420 nm in these samples using a Perkin-Elmer fluorescence spectrophotometer (Model LS-3) were carried out in quartz cuvettes. Calculation of GSH levels were made by relating the fluorescence intensities to a standard curve, obtained from replacing the sample volume with known concentrations of GSH, which were made up fresh daily. Standard final concentrations used were 5 μ M, 2.5 μ M, 1.25 μ M, 0.5 μ M, and 0.05 μ M, and GSH. Values were corrected for any quenching of fluorescence by 'spiking' the soluble fraction with a known concentration of GSH before assaying.

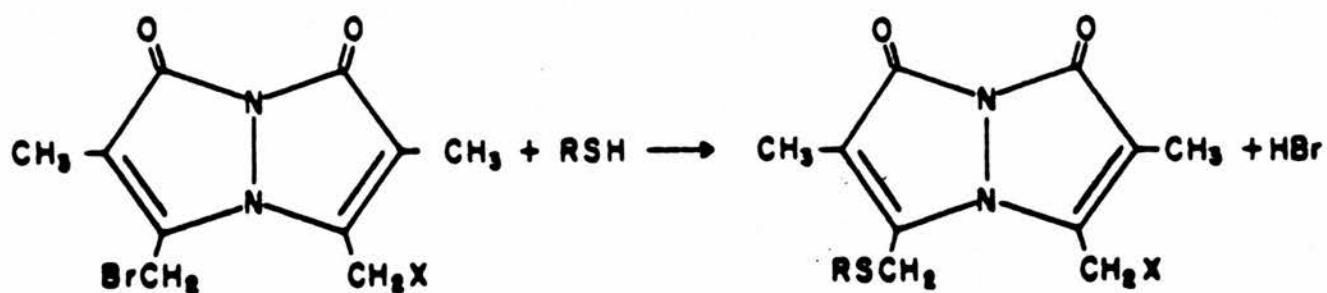
(b) H.p.l.c. assay using monobromobimane.

There are many different h.p.l.c. methods available for the determination of GSH. These include post or precolumn derivatisation of samples; use of different types of chromatographic resins and different detection systems. The assay used here involves the pre-column derivatisation of samples with a compound monobromobimane (MBBr) which reacts with thiols selectively to form a fluorescent derivative (Figure 9). This method is the most rapid, sensitive, and convenient h.p.l.c. method for thiol analysis and is based on the technique of Newton *et al* (1981) as modified by Gaetjens *et al* (1984).

(i) Preparation of samples.

Cells were harvested, as described in Section 2.1.3, washed twice in PBS, resuspended in 1ml of PBS, transferred to an eppendorf tube and sedimented at 18,000g average for 1 minute in an Eppendorf 5414 microcentrifuge. Supernatant was removed using a syringe and needle, 40µl of acetonitrile (h.p.l.c. grade, Rathburns, Peebles, Scotland) was added slowly to the cell pellet and the sample mixed using a 7mm magnetic bar. 15µl 0.5M Tris HCl, 3.6mM EDTA pH 7.5 was added, and 5µl of 30mM solution of MBBR (Calbiochem UK Ltd, Cambridge Science, Cambridge) in acetonitrile. Stirring was continued for 20 minutes under subdued lighting and the derivatisation terminated by the addition of 5µl of acetic acid. The assay mixtures were diluted to a volume of 1ml with the water-methanol-acetic acid elution buffer 'A' of Newton *et al* (1981). Buffer A contained 100ml methanol (h.p.l.c. grade, Rathburns, Peebles, Scotland) 2.5ml acetic acid, diluted to 1 litre with double distilled deionised water, titrated to pH 3.9 with 50% NaOH, and filtered through a 0.2µM millipore filter. This 1ml sample was centrifuged at 18,000g

FIGURE 9. Fluorescent labelling of thiols with monobromobimane
(mBBR, X = H) or monobromotrimethylammoniumbimane
(qBBR, X = $^+N(CH_3)_3$).



average for 20 minutes, filtered and 50 μ l of the supernatant subjected to h.p.l.c. analysis. Derivatised samples could be stored for several weeks at -80°C without loss of fluorescence.

(ii) High-performance liquid chromatography.

A Waters system consisting of two model 510 pumps, an automated gradient controller, a data module (Model M730) which was linked to a fluorescence detector (Model 420) fitted with a standard flow cell, and a 405nm filter, was used. The column was a waters Novapak C18 reverse phase (3.9mm x 150mm) coupled to a guard column, containing a disposable 3cm guard pak cartridge filled with μ bondapak C18 packing. The water-methanol-acetic acid elution programme used in the chromatography was as described previously by Newton *et al* (1981), but modified as follows: 0-15 minutes, 3% buffer B, isocratic, at 1ml/min, 15-17 minutes, 90% B, 17-25 minutes, 3% B (column regeneration). Buffer B contained 900 ml methanol (hplc grade), 2.5ml acetic acid and diluted and adjusted to pH 3.9 as previously described for Buffer A. Both buffers were filtered and degassed, using a sonic bath, before use.

A typical h.p.l.c. trace showing the GSH peak is illustrated in Figure 10. The GSH peak was identified by using a standard concentration of GSH which had been 'spiked' with tritiated ^3H GSH (10 μ l of 1MBq/ml) and also by showing that the peak increased in area with increased amount of standard concentration of GSH injected (Figure 11). This information was used by the data module to calculate the amount of GSH in an unknown sample relative to a standard concentration.

2.9.0 γ Glutamylcysteinylsynthetase (γ GCS) Assay.

The assay for γ GCS was carried out according to the method of Seelig & Meister (1984b), in which γ GCS activity was determined from the rate of

FIGURE 10. H.p.l.c. Trace of the Separation of MBBr derivatives.

Samples were derivatised and separated by reverse phase h.p.l.c. as described in Materials and Methods (Section 2.8.0). The chromatogram trace shown was typical of those obtained from cell lines (ovarian, PE01 cell line in this example). GSH-MBBr conjugate (GSH), MBBr hydrolysis product (H), and probably cysteine - MBBr conjugate (cys?) are indicated with retention times (mins).

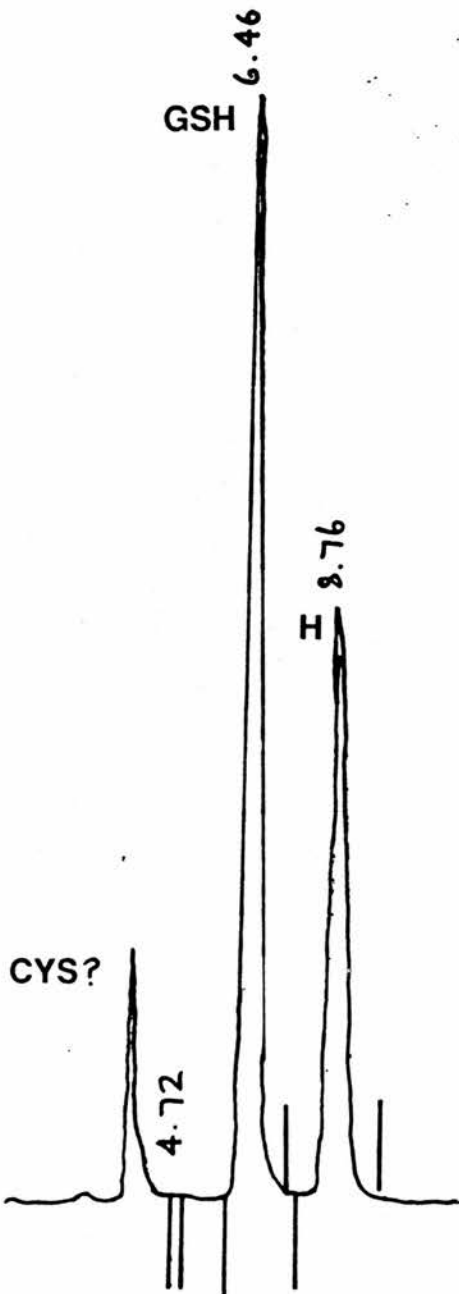
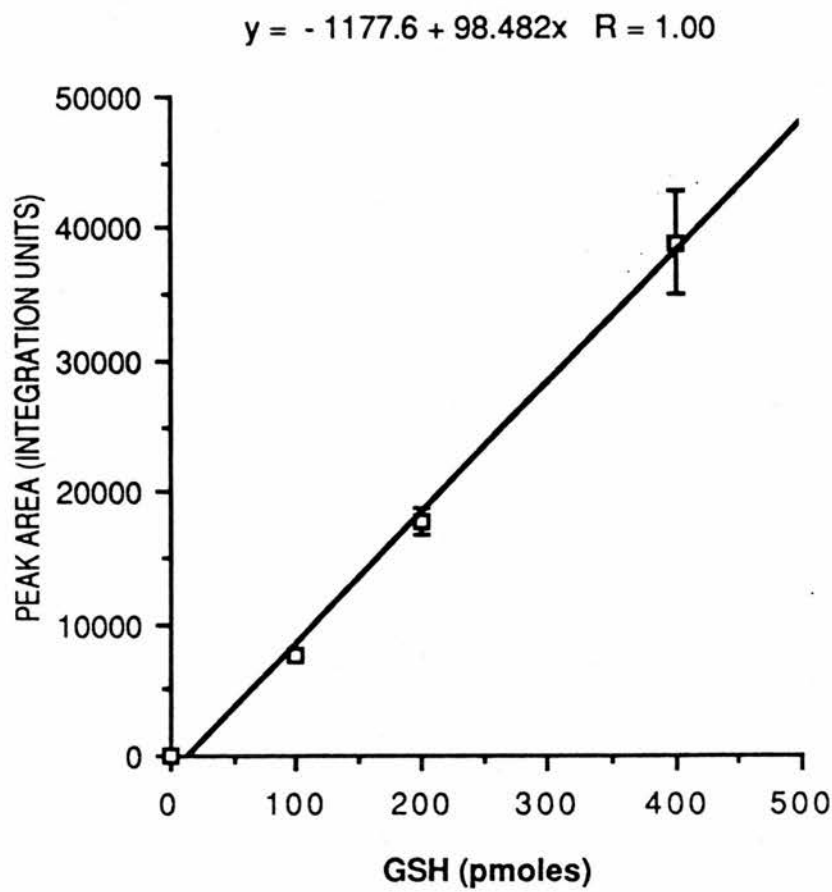
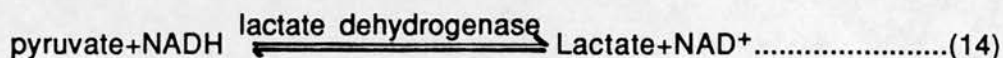
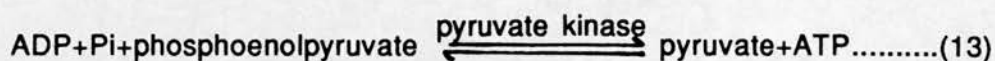
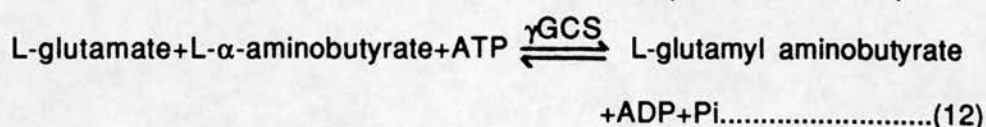


FIGURE 11. Graph showing Linear Relationships between
Peak Area and GSH Concentration.

The graph presented shows the integrated peak area calculated by the data module for GSH-MBBR peak, plotted against the amount of GSH derivatised injected onto the reverse phase h.p.l.c. column. For derivatisation and h.p.l.c. technique details see Materials and Methods (Section 2.8.0).



formation of ADP (assumed to be equal to the rate of oxidation of NADH) as calculated from the change in absorbance at 340nm (equations 12,13,14).



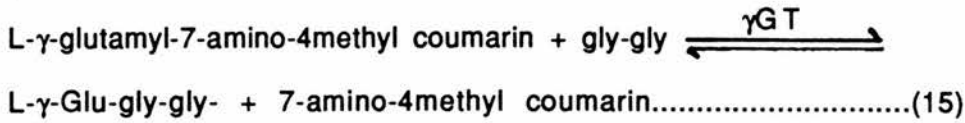
The final concentration of the components in the 1ml sample cuvette were as follows:

100mM Tris/HCl, pH8.0, 2mM EDTA, 150mM KCl, 20mM MgCl₂, 10mM L-glutamate, 10mM L- α -aminobutyrate (Sigma, Poole, Dorset), 0.2mM NADH (Sigma, Poole, Dorset) 17 μ g pyruvate kinase (Sigma, Poole, Dorset), 17 μ g lactate dehydrogenase (Sigma, Poole, Dorset) and 2mM mono(cyclohexylammonium) phosphoenol pyruvate. The reference cuvette contained the same components as the sample cuvette, except no soluble fraction was present. The change in absorbance at 340nm between these cuvettes, without ATP present, gave the background rate. Addition of 50 μ l of 100mM ATP to both the cuvettes gave the rate due to γ GCS activity present in the soluble fraction. Assays were run using a Schmidazu MPS 2000 spectrophotometer. The assay was verified by incubation of the soluble fraction with the γ GCS inhibitor buthionine-S-R-sulphoximine (10mM) (Sigma, Poole, Dorset) for 4 hours at 37°C prior to the assay. An almost complete inhibition in γ GCS activity was measured.

2.10.0 γ Glutamyltranspeptidase (γ GT) Assay.

Cell sonicates (prepared as described in Section 2.2) and tissue homogenates were used in the assay for γ GT activity, using the sensitive

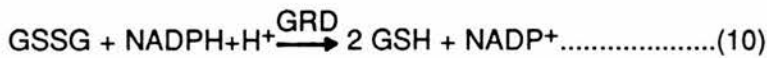
fluorometric method described by Smith et al (1979) using the substrate L- γ glutamyl-7-amino-4 methyl coumarin (equation 15).



Dilutions of the samples were made in 0.1m ammendiol/HCl buffer pH 8.6 (Ammendiol: 2 amino-2-methyl-1,3,propandiols, was obtained from Sigma, Poole, Dorset). 100 μ l of these dilutions were incubated at 37°C for 10 minutes with 250 μ l of 0.2mM γ glutamyl-7-amino-4-methyl coumarin as substrate (Universal Biologicals, Cambridge), diluted with ammendiol/glycylglycine/triton buffer (100ml ammediol buffer, 20mM glycylglycine 100 μ l triton X100) from a 10mM stock in 2-methoxyethanol (Aldrich Chemical Co., Gillingham, Dorset). The reaction was stopped by the addition of 2ml of ice cold 0.05M glycine buffer, pH 10.4, and fluorescence determined using a Perkin-Elmer Model LS-3 fluorimeter at excitation 370nm and emission 440nm. Values were quantitated from a standard curve, obtained using varying concentrations of the fluorescent product 7-amino-4-methyl coumarin (Sigma, Poole, Dorset) 0.2 - 20 μ M, diluted from a 10mM stock with ammendiol/glycylglycine/triton buffer. The assay was verified by incubation of the sample at 37°C for 10 minutes with γ GT inhibitor 5mM serine/10mM borate before assaying. This could almost completely inhibit all γ GT activity (90%).

2.11.0 Glutathione Reductase (GRD) Assay.

GRD activity (equation 10) was assayed in the soluble fractions by the fluorometric method of Weiss et al (1980).



The final concentration in the reaction mixture sodium phosphate buffer, 67mM pH 6.7, were 0.5mM NADPH, 2.0mM nicotinamide, 3.0mM GSSG and 10 μ l of 1-2mg/ml soluble fraction. The final volume was 40 μ l. The mixture was

incubated at 37°C for 15 minutes and the reaction stopped with the addition of 40µl of 0.1 N HCl. Addition of HCl also destroys any unoxidised NADPH (Lowry & Passonneau, 1972). Fluorescence of NADP⁺ was induced by adding 1.0ml of 6N NaOH containing 0.03% (v:v) H₂O₂ and heating the tubes to 60°C for 10 minutes. Fluorescence intensities were determined in Perkin-Elmer fluorometric spectrophotometer (Model LS-3) at excitation 360nm and emission 460nm. Concentrations of NADP⁺ 12.5µm, 25µm, 50µm, 100µm and 200µm were used to generate a standard curve to calculate NADPH oxidation rates and therefore GRD activity. A blank was included in the assay, consisting of reaction mixture minus the sample, and was subtracted from both the standards and the sample assays, before drawing the curve.

2.12.0 **Glutathione Peroxidase (GPX) Assays.**

2.12.1 Total GPX activity (Selenium and non-selenium dependent forms).

Total GPX activity in the soluble fraction of cells and tissues were determined by the method of Paglia & Valentine (1967) at 37°C using cumene hydroperoxide (CHP) as a substrate. Reaction buffer contained 100mM Tris/HCl pH, 7.2, 3mM EDTA, 1mM sodium azide, 1.2mM CHP, 0.5mM NADPH and 1 unit of glutathione reductase (Sigma, Poole, Dorset) in 1 ml final volume. 50µl of sample was added, and the oxidation of NADPH monitored at 340nm on a Shimadzu MPS 2000 spectrophotometer.

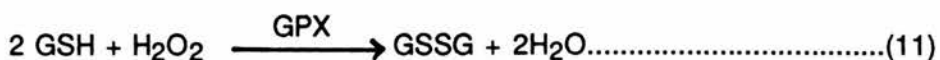
2.12.2 Selenium-dependent GPX activity.

Two different assays were used, the fluorometric method of Weiss et al (1980) and the spectrophotometric assay of Paglia & Valentine (1967).

(a) Fluorimetric assay.

This method was used initially only for determining selenium-dependent GPX activity in PE01, PE04 and MCF7 cell lines and was later substituted for the more convenient spectrophotometric assay. The procedure was similar to

that described in Section 2.11.0 for GRD. The reaction involved a coupled assay as indicated in equations 10, 11.



Final concentrations of the components in 0.5M sodium phosphate buffer, pH7.0, were 3.0mM GSH, 0.5mM NADPH, 2.0mM sodium azide, 4.0mM EDTA, 4.0mM nicotinamide, 2.0µg/ml GRD (Sigman, Poole, Dorset, 240 units/mg protein) and 5µl of 3mg/ml soluble fraction, in a volume of 40µl. Following a 10 minute preincubation period at room temperature, to activate the sulphhydryl enzyme, the reaction was initiated by adding H₂O₂ (final concentration 0.5mM). The reaction mixture was incubated at 37°C for 15 minutes, and terminated by the addition of 40µl of 1.ON HCl. Fluorescence of NADP⁺ was induced by adding 1.0ml of 6N NaOH containing 0.03% H₂O₂, and the tubes heated to 60°C for 10 minutes. Fluorescence was measured at excitation 360nm and emission 460nm. Activity was calculated from standard curves, in which the soluble fraction was replaced with 25µm, 50µm, 125µm, 250µm of GSSG and 10µm, 25µm, 50µm, 100µm of NADP⁺ respectively. A blank using the reaction mixture without soluble fraction present was subtracted from all the values. The rate of reaction with time was shown to be linear (Weiss et al 1980), and also the non-specific peroxide rate was found to be minimal.

(b) Spectrophotometric assay

This assay for selenium-dependent GPX activity was carried out essentially as described for total GPX activity, except CHP was replaced with 0.25mM H₂O₂ as the substrate.

2.12.3 Non-selenium dependent GPX activity.

Non-selenium dependent GPX activity was determined by subtracting the value for the selenium-dependent form from that for total GPX activity as

described by Masukawa et al (1983). This activity has been shown to be associated with alpha class GST subunits.

2.13.0 **Glutathione S-transferase (GST) Assays.**

2.13.1 Activity towards 1,4-dichloro-2,5-dinitrobenzene as a substrate.

Activity towards 1,4-dichloro-2,5-dinitrobenzene (CDNB) as a substrate was determined according to the method of Habig et al (1974b) (equation 16) (please see separate page). 41mg of CDNB (Sigma, Poole, Dorset) was dissolved in 4ml ethanol, and added, drop by drop, to prewarmed 0.1M sodium phosphate buffer pH 6.5 (196 mls). Aliquots of this buffer were put into both the reference and sample cuvettes, together with 1mM GSH (final concentration in 1ml volume) which was only added to the sample cuvette. The non-enzymatic background rate, at 340nm, was then measured. 10 μ l - 50 μ l of diluted or undiluted soluble fraction was added to the sample cuvette, and the enzymatic rate determined at 340nm. All measurements were carried out at 37°C, using a Shimadzu MPS 2000 spectrophotometer.

2.13.2 Activity towards ethacrynic acid (EA) as a substrate.

GST activity towards EA as a substrate was determined at 37°C according to the method of Habig et al (1974b), which monitored the formation of ethacrynic conjugate at 270nm (equation 17) (please see separate page).

The sample and reference cuvettes contained 0.1M potassium phosphate buffer, pH 6.5, to which EA was added to a final concentration of 0.2mM (final volume 1ml). EA was made up fresh in ethanol before use. GSH at a final concentration of 0.25mM was added only to the sample cuvette, and the non-enzymatic background rate monitored at 270nm. 50-100 μ l of soluble fraction was added to the sample cuvette and the rate determined.

2.13.3 Activity towards cumene hydroperoxide (CHP) as a substrate.

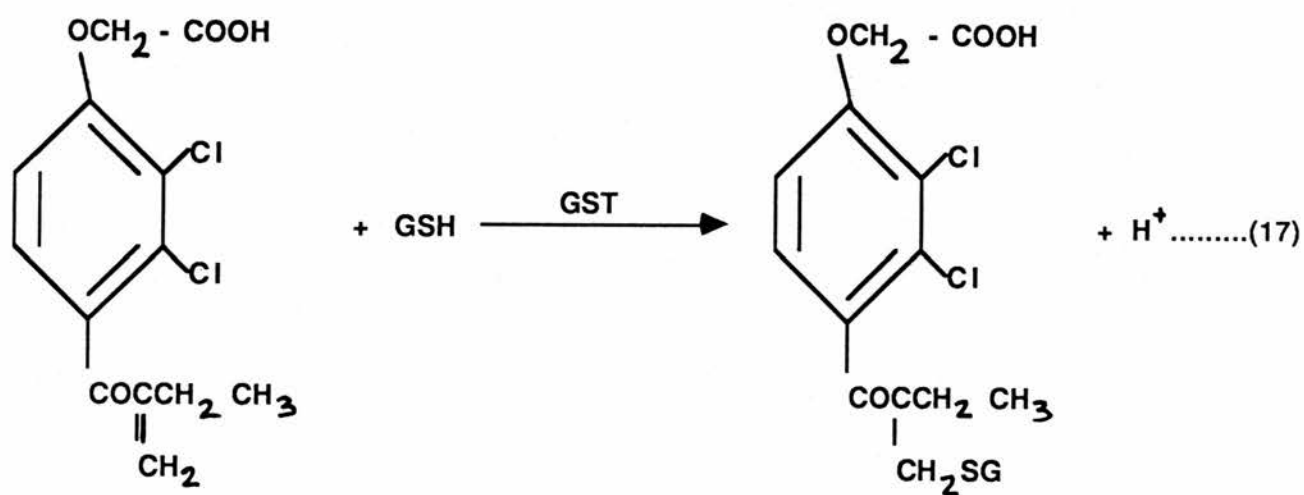
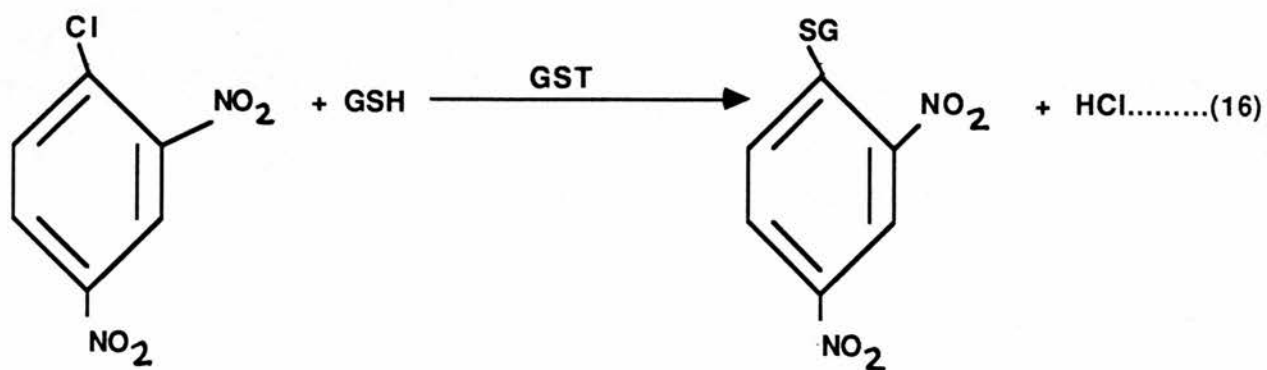
GST activity towards CHP as a substrate can only be used for purified proteins and not soluble fractions. Purified GST protein activity towards CHP

as a substrate was carried out as described in Section 2.12.1. CHP activity can be indicative however of alpha-class GST levels, as mentioned in Section 2.12.3.

2.14.0 **Statistics.**

Statistics, including degree of significance using the students T-test, were carried out using the Statsworks programme on an Apple Macintosh Plus computer.

Equations 16 and 17



**3.1.0 Glutathione S-transferase isoenzymes in human
 tumours and tumour derived cell lines.****3.1.1 Introduction.**

It is likely that glutathione S-transferase (GST) levels and isoenzyme composition will play a role in both the intrinsic and acquired resistance of tumours to cytotoxic drugs. As a consequence, it is important to establish the constitutive expression of these proteins in a variety of tumours and tumour derived cell lines in order to provide information regarding their possible responses to cytotoxic drugs. In this regard, Batist et al, (1986) have shown that in a breast carcinoma cell line (MCF7), which was multidrug resistant to adriamycin, a 40 fold elevation in a pi class subunit was found, but how significant is this finding? Evidence from the GST subunit composition of other cell lines may help to clarify this situation. This information would also be useful with respect to the selection of recipient cells for transfection experiments using expression vectors containing clones encoding for specific GST subunits. This type of experiment would give valuable data regarding both the regulation, and the effectiveness, of a specific GST subunit in drug detoxification, and therefore a role in drug resistance.

Tumour derived cell lines are used as models for solid tumours, especially in the screening of new anticancer drugs. Information concerning the differences or similarities of GST subunit composition between the tumours and tumour derived cell lines from the same tissue source would be of interest in establishing the validity of this cell line model. It is important to remember, however, that there are other limitations in using cell lines as models for tumours, including the differences in morphology and characteristics of cells in culture.

In the study described here, in order to characterise the GST's in human tumour tissues and cell lines, both total GST activity and subunit composition in a series of six different human tumours and nine cultured human tumour cell lines have been studied.

3.1.2. Results.

GST activity, using CDNB as a substrate, in nine human cell lines of different tumour origin, and a mouse hepatoma cell line Hepa 1, is shown in Figure 12. A large variation in activity between the lines was observed. The breast cancer cell line, MCF7, had by far the lowest activity (3.5 nmoles CDNB conjugated/min/mg protein), which was approximately 60 fold lower than the ovarian adenocarcinoma cell line PE04 (210.3 nmols CDNB conjugated/min/mg protein). The human clara cell derived lung line NCI H358, foetal lung EF484, and the colon line HT29, all had high GST activity. A second group of cell lines, consisting of the colon line, LS174T; bladder, EJ; liver HepG2 and lung NCI H322, all had moderate GST activity. The mouse hepatoma line Hepa 1 had activity in the range between the high and moderate GST containing groups of cell lines. This difference in GST activity, more markedly seen between PE04 and MCF7, was also reflected in the GST subunit content. The pi class subunit (λ) was expressed in most of the cell lines (Figure 13). The expression of this protein appeared to fall into two groups; cell lines which expressed high levels of this subunit, and those where this enzyme was present at very low levels, i.e. the MCF7, Hepa 1 and HepG2 lines. Slight differences were also observed in the pi class subunit expression in the cell lines containing high levels of this GST. All the cell lines contained proteins which reacted with an antibody raised against the alpha class (B_1) subunit. However, the level of this protein was very low and could be only seen after developing the autorad after 96 hours exposure. Interestingly, the human hepatoma line HepG2 was the only line to contain high levels of this subunit. In most of the cell lines the mu class (μ) GST was present at very low concentrations and could only be identified on 96 hours

FIGURE 12. GST Activity In Tumour Derived Cells.

Cell lines were derived from human tumours except HEPA1, which was a mouse hepatoma cell line. 10⁷ cells were harvested from confluent cultures with trypsin:versene, and prepared as described in Materials and Methods (Section 2.2.0). GST activity was measured using CDNB as a substrate (Habig *et al*, 1974). Values are expressed as nmoles CDNB conjugated/min/mg protein and are means from three separate experiments. Tumour tissue source is indicated above the cell line value. The different cell lines were indicated as: P = PE04, M = MCF7, E = EF484, EJ = EJ, H2 = NCI H322, HS = NCI H358, L = LS174T, H = HEPA1, HEP = HEPG2, HT = HT29 and S = Standard.

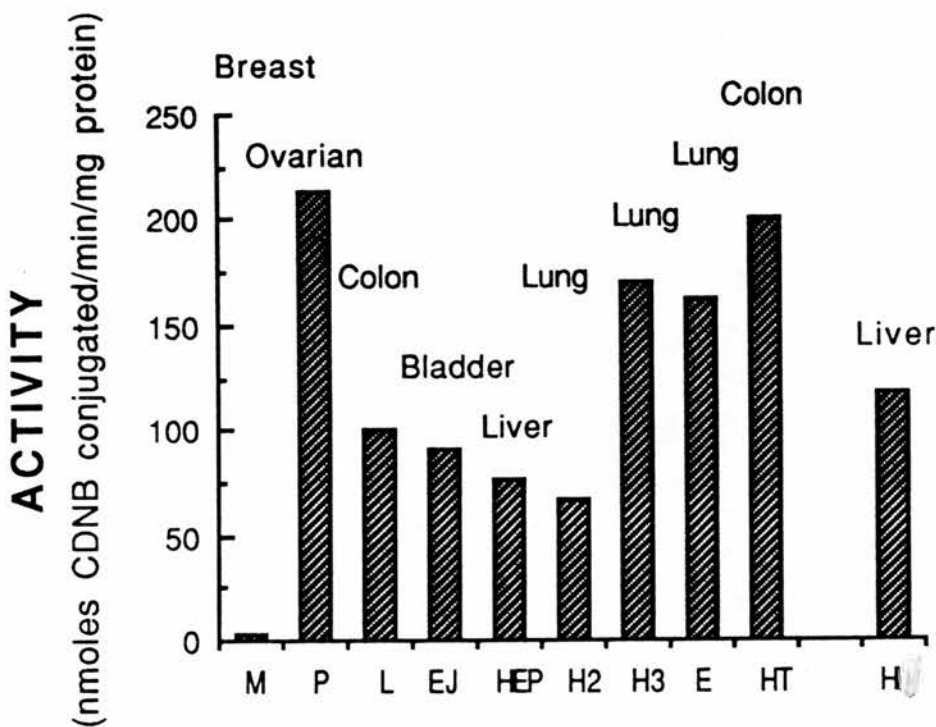
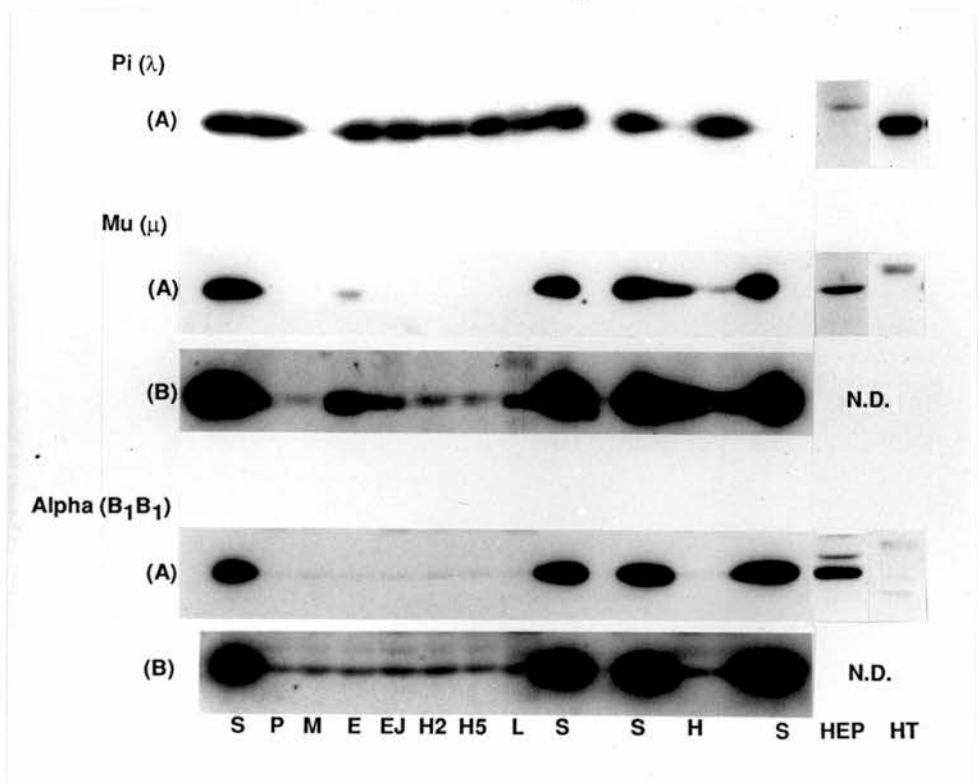


FIGURE 13. Glutathione S-transferase Subunit Content In
Tumour Derived Cells.

10⁷ cells were harvested from confluent cultures with trypsin:versene, and prepared as described in Materials and Methods (Section 2.2.0). 50µg of soluble protein with the exception of HT29 (25µg) was ran out on SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against the three known human GST isoenzyme classes: Pi (λ), alpha (B₁), and mu (μ). The different cell lines were as indicated in legend to Figure 12. The exposure times before developing the autorads were as indicated a) 12 hours; b) 96 hours; ND, not determined.



exposure of the blots. The Hepa 1, the human lung fibroblast EF484 and HepG2 lines, contained significantly higher levels of this subunit relative to the other lines tested.

Differences in GST subunit profiles between cell lines and tumours derived from the same tissue source was observed (Figure 14). Interestingly, the human breast tumours contained high levels of the pi class (λ) subunit, in contrast to the cell line. The expression of this GST was however high in the majority of tumours and the homologous cell line. The concentration of this isoenzyme in both the human hepatoma and normal liver tissue was low, but present at levels higher than that found in the hepatoma cell line. Alpha class (B_1) GST was found at high levels in the lung, ovary, and liver tumours, and also in the normal liver sample, as expected. A low level of this polypeptide was observed, however, also in the bladder tumour. The mu class (μ) protein was seen at high concentration in only one breast tumour, the bladder, the liver tumour and normal liver tissue samples. Low levels were detected in the lung and the ovarian tumours. This concentration of the alpha and mu class subunits in the tumours followed the cell line levels to some degree.

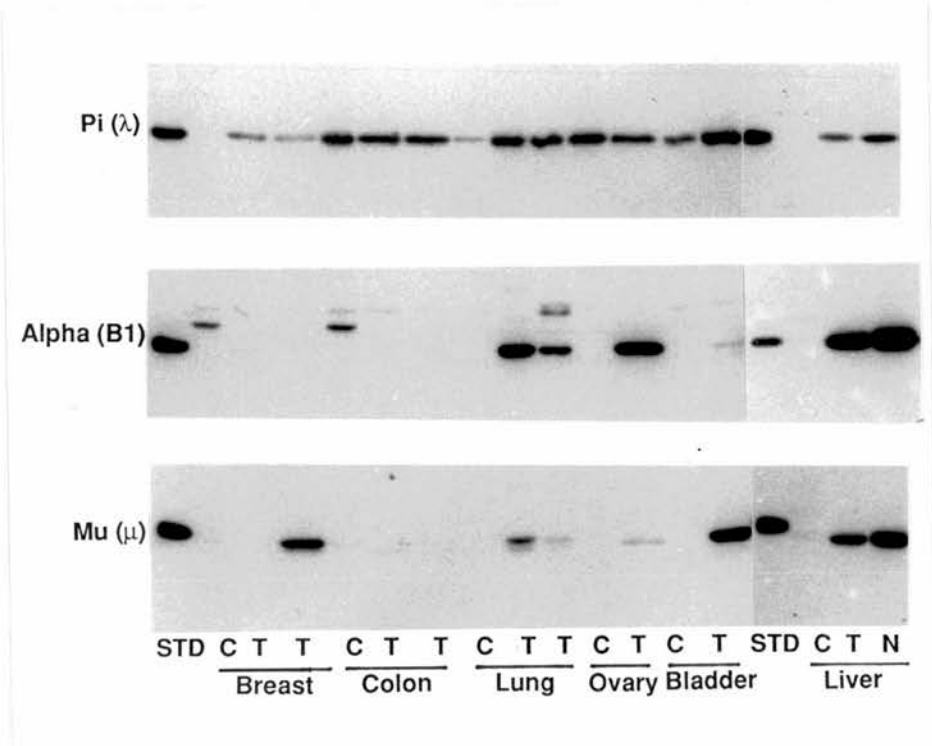
3.1.3 Discussion.

Cell lines are used extensively for both the screening of new anticancer drugs and for the investigation into their mechanism of action. The reason for this is that large amounts of tumour samples are often difficult to obtain, and cell lines are easier to manipulate than tumours with regard to drug treatment. It is important to remember, however, that cell lines derived from tumours may change their phenotype and characteristics in culture, compared with the original tumour, and therefore may no longer be representative of the tumour, in addition to any changes in GST composition which may occur.

In view of the role of GST's in detoxification reactions, I have tried to establish whether the detoxification capacity of a variety of commonly used cell lines of different tumour origins was representative of that measured in

FIGURE 14 Glutathione S-transferase Subunit Content in Tumour Derived Cell Lines and Tumours derived from some Tissue source.

10⁷cells were harvested from confluent cultures with trypsin:versene and prepared as described in Materials and Methods (Section 2.2.0). Normal and tumour tissues were prepared in collaboration with Dr. L. Forrester. 50µg (alpha and mu blot) or 25µg (pi blot) of soluble protein were ran out on SDS/PAGE, transferred to nitrocellulose and probed with specific antibodies raised against either alpha class (B₁), mu class (µ) or pi class (λ) subunits respectively. Profiles were typical examples of the tumour types. Abbreviations used are: C = cell line; T = tumour; N = normal tissue; STD = standard. Tissue source is as indicated.



the original solid tumour, and also whether these cell lines will prove useful for understanding the function and regulation of the GST's. (The soluble fraction from the cell lines and the boiling mixes were prepared by myself, but the soluble fractions from the tumours, and also the Western blot, were carried out in collaboration with Dr. L. Forrester in our laboratory). Most of the solid tumours studied contained high levels of the pi class GST (λ) subunit, with mainly small variations seen between individuals, in agreement with reports by Kodate et al, (1986) for colon carcinoma and Shea et al, (1988) for breast and renal carcinomas. The content of this enzyme in cell lines was very similar to the tumour content, even in the liver samples where the hepatoma and hepatoma cell line had low levels of this protein. The only exception was the MCF7 cell line, which contained a much lower level of the pi class subunit than the tumour samples. In terms of GST content and activity, this cell line would not appear to be a model for breast tumours in their susceptibility to anticancer drugs. Batist et al (1986), in his multidrug resistant cell line, would, therefore, have only increased GST levels, and expression of the pi class protein approaching that of most other cell lines and tumours.

The tumour levels of the mu and alpha class GST subunits is subject to considerable individual variability (Carmichael et al, 1988). For example, in lung cancer the tumour content of these enzymes appears to some degree to segregate according to tumour type (Carmichael et al 1988). It is, as a consequence, probably not possible to use specific lung cell lines to be representative of a particular tumour type. In contrast, the human hepatoma cell line HepG2 has been used to study normal gene expression and function, such as bile acid biosynthesis (Everson & Polokoff, 1986) and apolipoprotein secretion (Zannis et al, 1981). GST's have been shown to be involved in bilirubin transport and consequently the profile of the subunits detected in the hepatoma cell line would be important in determining whether this line was indeed a good model at looking at normal liver functions. The HepG2 cell line

was found to contain levels of GST subunits similar to those found in normal liver (Figure 14; Warholm et al., 1983; Stockman et al., 1985; Hussey et al., 1986). On the basis of this finding, the cell line may prove extremely valuable for the study of the response of normal liver to cytotoxic and carcinogenic compounds, and also of the factors which regulate hepatic GST levels in man. The individual, however, from which this line was derived, would not appear to be null for the mu class (μ) protein, as this polypeptide was present in high concentration. In fact, all of the cell lines contained this enzyme, and also most of the tumours, and thus indicates that this polypeptide was not absent from these individuals as well.

Transfection experiments, using GST expression vectors into recipient cell lines, may only be carried out if the GST subunit composition is known. Therefore, the results obtained above may allow these investigations to be undertaken, and provide information about the role of these enzymes in drug detoxification in cell lines. The differences in GST subunit profile between tumour derived cell lines obtained from a variety of tumour sources, and between cell lines and solid tumours, indicates that their response to cytotoxic drugs may be different. The use of tumour derived cell lines as models for solid tumours must only be used with caution as a consequence.

3.2.0 Acquired drug resistance in tumour cell lines, generated in vivo and in vitro.

3.2.1 Introduction.

There are very few studies carried out where glutathione S-transferases and other glutathione-dependent enzymes have been determined in drug resistant cell lines. In the study described here, glutathione, and a range of glutathione-dependent enzymes, have been characterised in three drug resistant cell line models. These are a) a Chinese Hamster Ovary (CHO) cell line, made resistant to a nitrogen mustard, chlorambucil; b) an in vivo model, where two ovarian adenocarcinoma cell lines were derived from a single patient

patient before (PE01) and after (PE04) the onset of drug resistance and c) a human sarcoma cell line which has the typical multidrug resistance (MDR) phenotype.

(a) Chlorambucil resistant CHO cell line.

In a previous report, a chlorambucil resistant CHO cell line resistant to other bifunctional nitrogen mustards was described (Robson et al. 1986). The chlorambucil resistant cell line was selected by growing cells for 3 months in the presence of progressively higher concentrations of chlorambucil. In particular, the cell line was resistant to chlorambucil (24 fold) and cross resistant to mechlorethamine (35 fold) and melphalan (13 fold). No resistance was observed to other bifunctional alkylating agents which generate DNA cross links via a reaction at the O⁶ of guanine, or to drugs like adriamycin. A cytosolic protein, with a molecular weight of approximately 25,000, and an isoelectric point of around 7.5, was overexpressed in the drug resistant line CHO-Chl^r. There was no change in the accumulation of the drug, indicating that the resistant cell line did not express the MDR phenotype (Robson et al. 1986), and also no difference in DNA repair in the resistant cell line was observed (Robson et al. 1987). In the study described below the possibility that an increased rate of drug detoxification as a consequence of elevated GSH and glutathione-dependent enzymes including the GST's was investigated.

(b) In vivo drug resistant cell line model.

A significant problem in the study of drug resistance is establishing the relevance of in vitro experiments, such as those indicated above, to clinical observations in vivo. In this respect, two ovarian adenocarcinoma cell lines were derived from a patient before (PE01) and after (PE04) the onset of drug resistance to a combination of cis-platinum, chlorambucil and 5-fluorouracil (Wolf, et al. 1987b). The PE01 cell line was, in fact, derived from ascites fluid obtained after the relapse of a patient from initial chemotherapy. A complete response was achieved however using the same previous course of treatment.

Three months later the patient relapsed again, but this time the patient no longer responded to treatment, including high dose cis platinum. The PE04 cell line was derived from this second ascites sample. Using these cell lines it is possible to establish whether differences in glutathione-dependent enzymes are present which could, therefore, be an important factor in the clinically observed acquired drug resistance.

In this model I have also studied whether detoxification enzymes are modulated during the cell cycle, as there is evidence which shows altered sensitivity of cells in logarithmic growth to alkylating and chemotherapeutic agents, such as platinum compounds etc. (Frei & Harsano, 1967; Watanabe & Harikawa, 1975; Edwards & Nias, 1986). As a consequence, the regulation of glutathione-dependent enzymes during cell division, to establish whether this may be a factor in the differential sensitivity to cytotoxic drugs, has been undertaken in these lines.

(c) Human sarcoma cell line showing multidrug resistant phenotype.

The development of broad cross-resistance to cytotoxic drugs has been observed recently in several mammalian lines, including the human sarcoma line DX5 (Harker & Sikic, 1985). Batist, et al., (1986) has shown, however, there can be simultaneously more than one mechanism of resistance in cells made resistant to anticancer drugs, with the over expression of GP180 and GST's found in his MDR MCF7 cell line. It is of interest, therefore, particularly in the case of the DX5 cell line, to look at the role of GSH and GSH-dependent enzymes in the resistance mechanism, as the degree of resistance in this line cannot be accounted for by increased expression of P-glycoprotein (GP180) alone (Sikic, et al. 1988). The possibility exists that there is a relationship between GP180 and GST's, as Thorgeirsson, et al. (1987) and Sikic, et al. (1987 unpublished) have shown that GP180 is overexpressed in hepatocarcinogenesis and regenerating rat liver, two models in which GST's are

also overexpressed. Whether this occurs in drug resistant cell lines, as indicated by Batist, et al. (1986), has to be shown more conclusively.

3.2.2 Results.

(a) Chlorambucil resistant CHO cell line.

Glutathione S-transferase (GST) activity, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, was measured in the wild type (CHO-K1) and resistant (CHO-Chl^r) cells. Table 5, Figure 15, showed that the CHO-Chl^r cells had a 2.7 fold higher total GST ($p < 0.02$) and a 1.8 fold higher GSH (carried out by Dr. A. Hall) content than the wild type line. In order to identify which of the GST isoenzyme subgroups were overexpressed, assays were carried out using substrates which show a degree of specificity for the pi, alpha (B₁) and mu gene families, (see Table 3) although care must be noted with respect to use of these substrates for CHO GST's. No activity towards styrene oxide (a marker for the mu class isoenzyme) was found. In contrast, however, significant elevations in cumene hydroperoxide (5.2 fold, $p < 0.005$) and ethacrynic acid (1.9 fold $p < 0.07$) activities were seen. These activities are indicative of the alpha and pi class GST isoenzymes respectively, although more than one class of GST may have activity with these substrates in the Chinese hamster (see Table 3). The elevation in glutathione peroxidase activity towards cumene hydroperoxide appeared to be completely due to differences in GST expression, as the selenium-dependent activity measured using hydrogen peroxide (Table 5, Figure 15) was similar in both lines. Of other glutathione-dependent enzymes involved in maintaining reduced GSH levels within cells, glutathione reductase (GRD), γ -glutamyltranspeptidase (γ GT) and γ -glutamyl-cysteinylsynthetase (γ GCS), only γ GT was significantly elevated (3.5 fold, $p < 0.005$) in the drug resistant line CHO-Chl^r. Evidence from the enzymatic analysis indicated that alpha GST's were overexpressed in the CHO-Chl^r cell line. In support of this, the protein staining pattern of cytosolic samples from both the wild type and drug resistant line clearly showed (Figure 16) an

TABLE 5 Glutathione-dependent enzyme expression in CHO-K1 and CHO-Chl^r Cell Lines.

Cell line	Activity							
	GSH	GST			GPX	γ GT	γ GCS	GRD
		CDNB	EA	CHP				
CHO-K1	18.4+2.2	239+36	19.7+3.2	16.4+2.0	8.9+4.8	0.82+0.26	70.0+16.2	96.5+23
CHO-Chl ^r	33.0+5.6	638+115+	38.0+12.1+	83.4+18.3*	10.0+2.7	2.85+0.48*	97.7+51.8	129.5+61.5

The abbreviations used are: GSH, glutathione; GST, glutathione S-transferase; GPX, glutathione peroxidase; γGT, glutamyltranspeptidase; γGCS glutamyl cysteinyl synthetase; GRD, glutathione reductase; CDBN, 1-chloro-2,4-dinitrobenzene; EA,ethacrynic acid ; CHP cumene hydroperoxide. All reactions were carried out at 37°C. Values are expressed as nmoles/min/mg protein; for CDBN, CHP, EA, H₂O₂, γ GCS and GRD or nmoles/min/10⁶ cells for γGT. GSH content is expressed as nmol/mg protein.

^{*} p < 0.005., ⁺ p < 0.01., ⁺ p < 0.07.

FIGURE 15. Relative Expression of Glutathione-Dependent
Enzymes In CHO-Ch1^f Cells.

The CHO-Ch1^f values shown are expressed as percentages of the CHO-K1 wild type line. See Table 4 for further details.

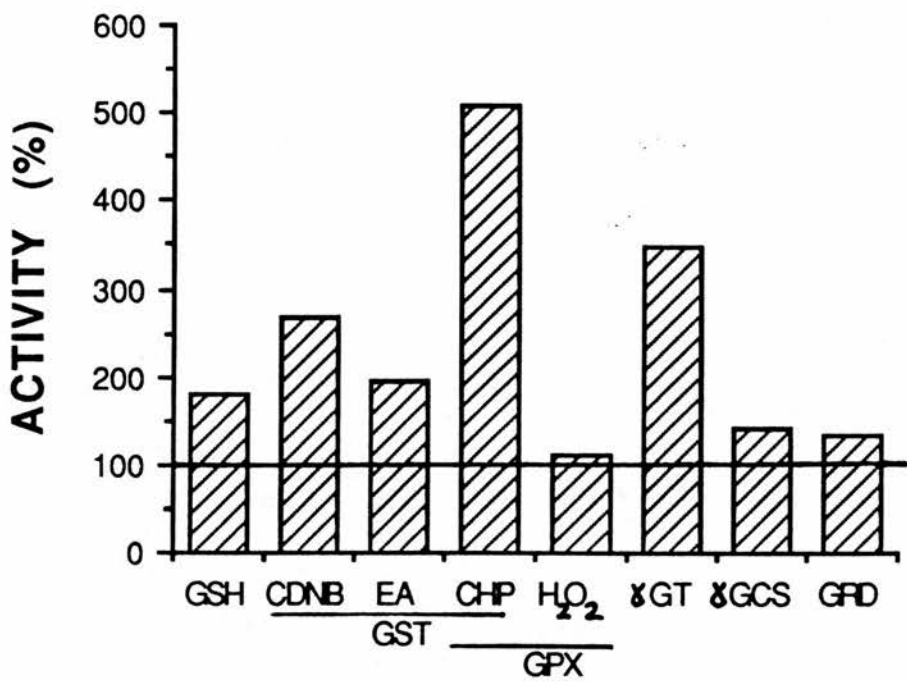
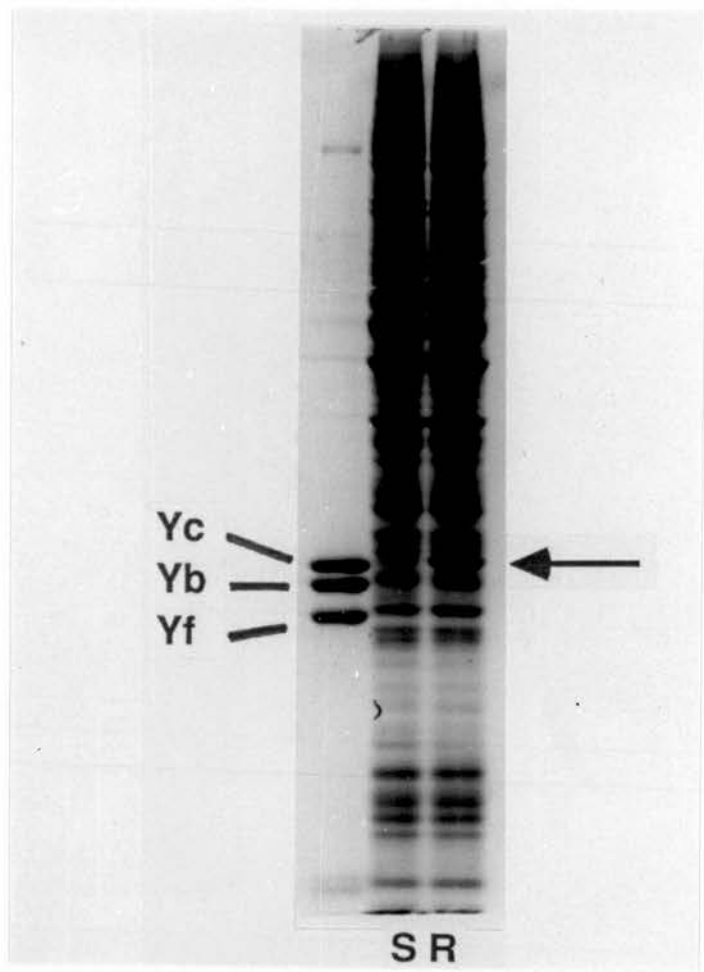


FIGURE 16 Protein Staining Pattern of CHO-K1 and CHO-ChI^r

Samples were separated by SDS-PAGE. 50µg of soluble protein was loaded per track. The standards shown are the rat subunits molecular weights 28500 Yc, 27000 Yb₁, 24500 Yf. K1 and ChI^r are the wild type and resistant cell lines respectively.



elevation in a protein with the same mobility as the rat alpha class Yc GST subunit in the resistant CHO-Chl^r line. Western blot analysis confirmed a dramatic increase of the alpha class GST subunits (Figures 17,18). Both the alpha, Ya and Yc subunits in the CHO-Chl^r cell line, appeared to be expressed at higher levels. A slight increase in the pi and mu proteins was also observed (Figure 17). In the blot shown in Figure 17, the antibody raised against the human alpha (B₁) GST subunit only reacts weakly with CHO alpha Yc subunits. The increase in the expression of the Yc subunit on the other hand, was easily seen when an antibody raised against the rat Yc protein was used (Figure 18). A forty fold induction of this protein, determined by densitometer scanning, was found in the CHO-Chl^r cell line compared with the wild type CHO-K1 line. The antibody raised against the rat Ya subunit did not react strongly with the CHO homologue (Figure 18), although an elevation in this subunit was again observed in the resistant cell line, in agreement with Figure 17.

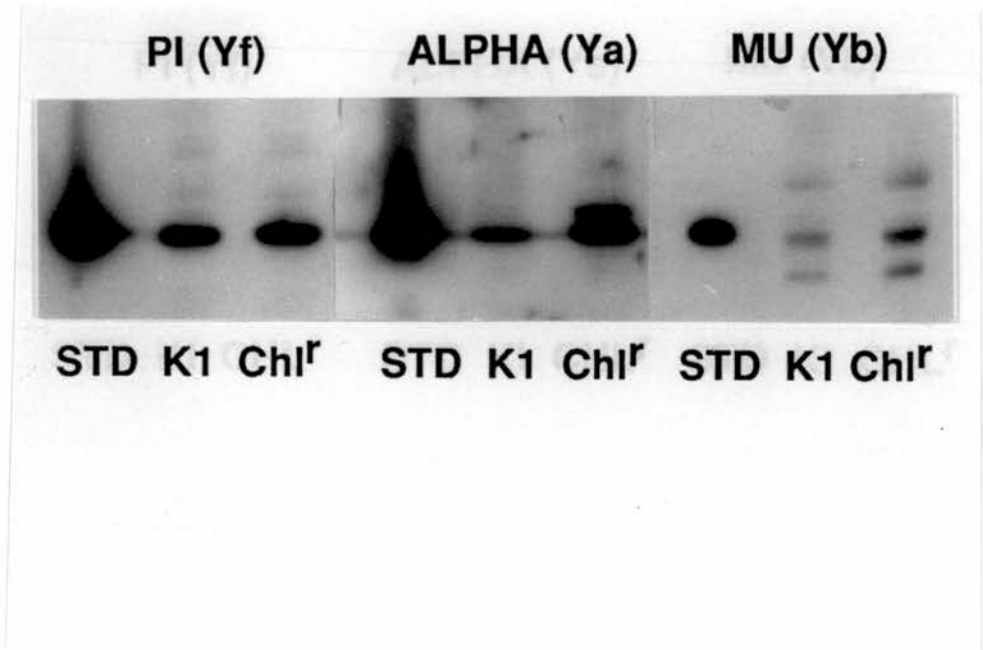
A protein was isolated by Dr. Ian Hickson from the resistant CHO-Chl^r cell line. This protein was found to have identical mobility to the alpha class rat Yc subunit on Western blot analysis (Figure 18). No cross reactivity of this purified protein was found with either antibodies raised against the human pi class, or mu class proteins (data not shown). Specific activities of this purified protein towards CDNB, ethacrynic acid, cumene hydroperoxide and hydrogen peroxide (Table 6) indicated that this protein is likely to be of the alpha class GST's (Table 3, Mannervik, 1985) providing Chinese hamster GST's have similar substrate specificities. No activity was detected with hydrogen peroxide as a substrate using the purified protein.

(b) Drug resistant ovarian lines generated in vivo.

Measurement of GSH levels and glutathione-dependent enzymes in drug sensitive (PE01) and resistant (PE04) cell lines, were initially carried out on confluent cultures (Table 7, Figure 19), and in all cases where differences were found, the PE04 cell line had the higher activities. GSH levels were

FIGURE 17 Glutathione S-transferase Subunit Content in CHO-K1
and CHO-Chl^r

Western blots were carried out as described in the Materials and Methods (Section 2.6.0) using 50µg of soluble protein per track. The bands were identified using specific antibodies raised to the human alpha class (B₁) and mu class (µ) subunits. STD represents human GST standard. K1 and Chl^r are the wild type and resistant cell line respectively.



**FIGURE 18 Comparison of Gutathione S-transferase Subunit
Content in CHO-K1 and CHO-Chl^r and the Purified
Protein.**

Western blots were carried out as described in the Materials and Methods (Section 2.6.0). Yc and Ya are rat standards; K1 and Chl^r are the wild type and resistant cell lines respectively. P is the GST protein purified from Chl^r. Antibodies used were specific to the alpha class rat Ya and Yc subunits (anti Yc and anti Ya).

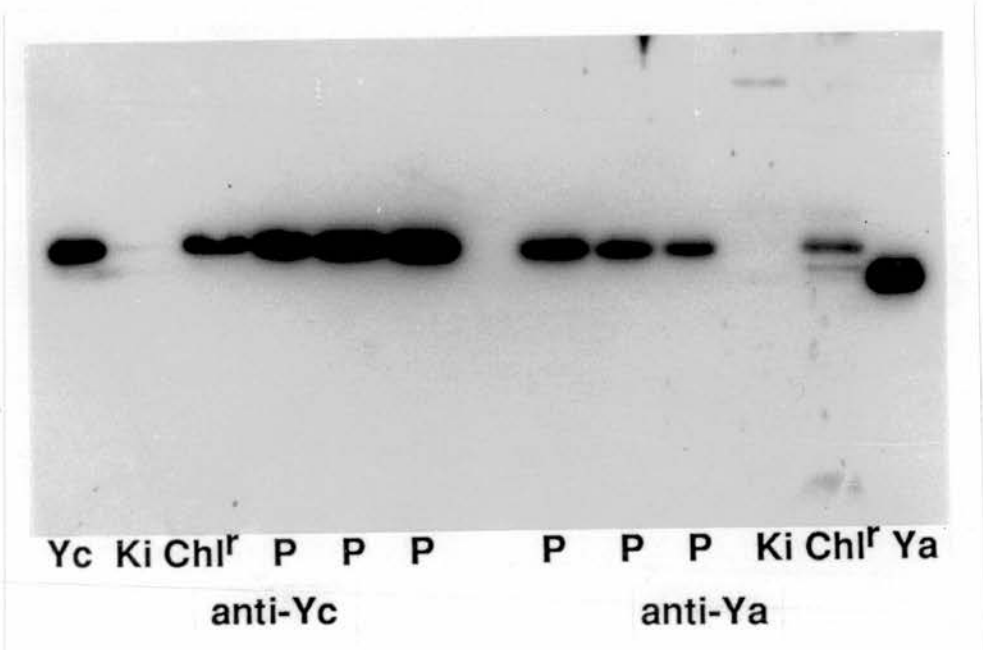


Table 6 Specific activity of purified GST towards
model GST substrates.

Substrate	Specific activity ^a μ mol/min/mg.
1,Chloro-2,4-dinitrobenzene	6.0
Ethacrynic acid	0.5
Cumene hydroperoxide	1.0

^a activity of the purified protein from CHO-Chl^r cells with each of the substrates was performed as described in Materials and Methods (Section 2.13.0).

Table 7
Glutathione Levels and Glutathione-Dependent Enzyme Activities
in Logarithmic and Confluent Cultures of PE01 and PE04.

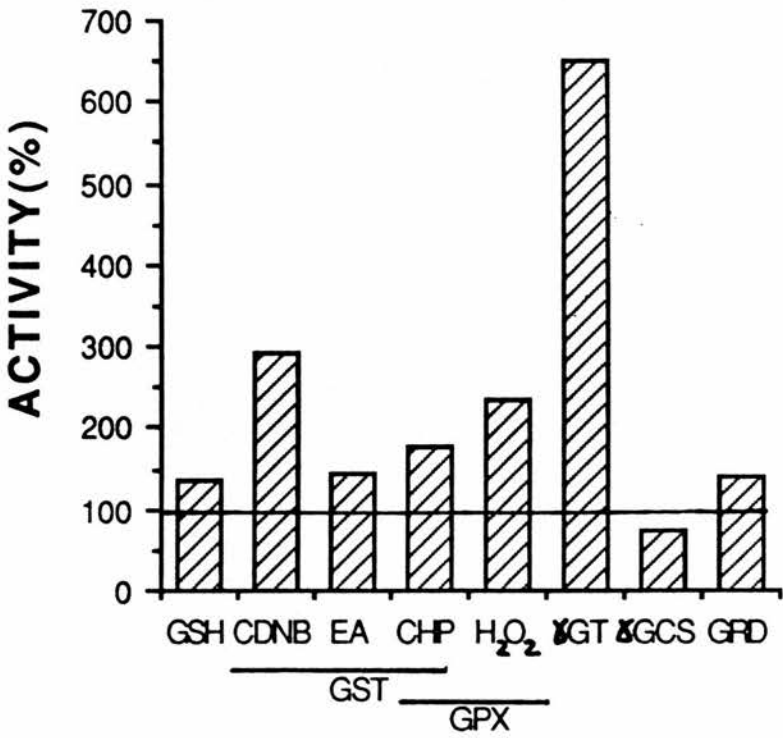
		ACTIVITY					
Thymidine Incorporation		GSH	γGCS	γ GT	GRD	GST	GPX
PE01 C	3805±387	27.8±6.7	16.8±0.64	0.06±0.01	62.3±5.8	74.0±9.3	11.6±0.7
L	12236±1232	42.9±3.3**	23.1±5.6**	0.08±0.03	62.9±5.0	95±30	38.9±1.9 *
PE04 C	29577±6174	37.4±5.2++	12.2±0.8	0.39±0.04+	87.4±12.3	215±15+	27.0±0.8+
L	72682±12260	77.7±19.4*	23.6±4.7*	0.33±0.07	89.5±16.2	213±39	52.1±2.1*

Thymidine incorporation is expressed as cpm per mg protein. GSH concentration is expressed as nmol/mg soluble protein. Enzyme activities are expressed as nmol/min/mg protein with the exception of γGT which is expressed as nmol/min/106 cells. Other details are given in Materials and Methods (Section 2.0).

Abbreviations used are γ GCS, γ-glutamylcysteinyl synthetase; γGT, γ-glutamyltranspeptidase; GRD, glutathione reductase; GST, glutathione-S-transferase measured using CDNB; and GPX, glutathione peroxidase measured using H2O2. C and L are confluent and logarithmic cultures, respectively. The values are shown as means ± standard deviations from three separate experiments carried out in triplicate. * Significantly different from confluent culture. p <0.001. ** Significantly different from confluent culture. p<0.01. + Significantly different from PE01. p<0.001. ++ Significantly different from PE01. p<0.01.

FIGURE 19 Comparison of Glutathione-Dependent Enzyme
Activities between PE01 and PE04 In Confluent
Cultures.

The PE04 values shown are expressed as a percentage of those obtained for the PE01 line. See Table 6 for further details.



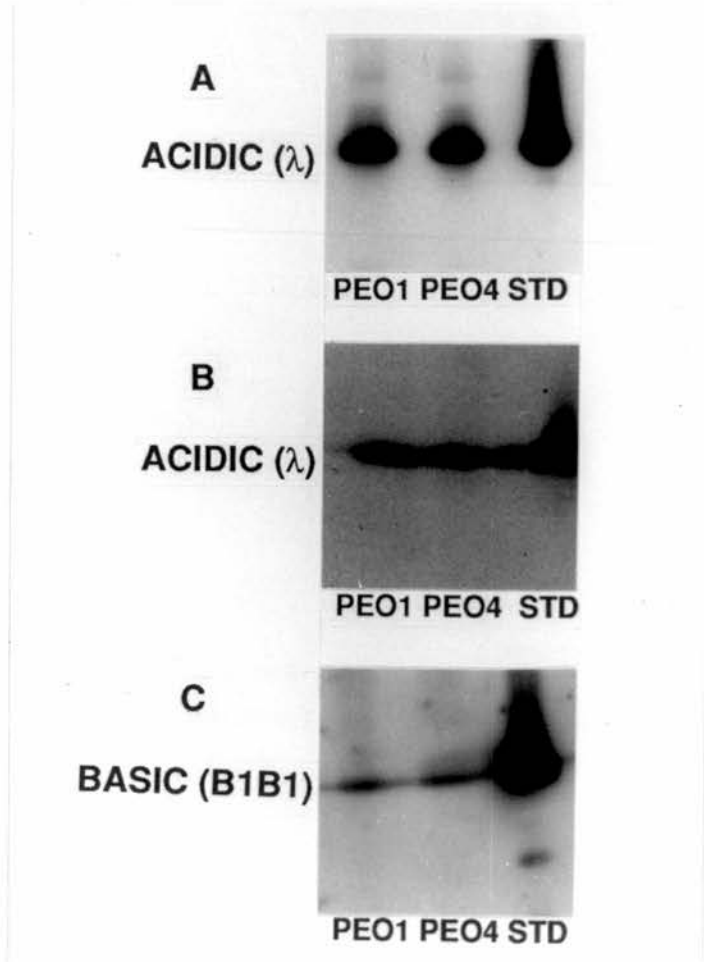
significantly higher in PE04 (1.4 fold, $p < 0.01$) and concomitantly with this, a 6.3 fold ($p < 0.001$) increase in γ GT activity was also observed. Other glutathione-dependent enzymes were studied in these lines. Increases in GST activity (1.9 fold, $p < 0.001$) towards the general substrate CDNB, and selenium-dependent glutathione peroxidase activity towards hydrogen peroxide, (2.3 fold, $p < 0.001$) were also found in the resistant line. The activities, however, of GRD and γ GCS enzymes were essentially unchanged in both of the cell lines studied. In addition to the increase in GST activity towards CDNB, the metabolism of other GST model substrates ethacrynic acid and cumene hydroperoxide were also carried out, and were elevated. The activities being 40.0 ± 6.8 and 58.4 ± 3.4 nmol/min/mg for ethacrynic acid and 14.6 ± 5.4 and 26.1 ± 8.1 nmol/min/mg for cumene hydroperoxide for PE01 and PE04 respectively.

In order to establish the GST subunit composition of the two ovarian adenocarcinoma cell lines, and also to determine whether the differences in GST activity towards CDNB, ethacrynic acid, and cumene hydroperoxide in the PE04 cell line could be associated with differences in the expression of known GST subunits, Western blot analysis was carried out.

Both cell lines were found to contain high levels of the pi GST when an antibody raised against the human pi class (λ) subunit was used (Figure 20) in the Western blot analysis. The human alpha class (B_1) subunit could also be detected in both lines, and was expressed on the blots at approximately 1/16th of the amount of the pi class protein. In either line, however, the mu class (μ) protein could not be detected at any significant amounts. The content, therefore, of the pi class (λ) and alpha (B_1) type subunits were similar in both lines, although the level of the B_1 subunit did appear to be slightly higher in the resistant PE04 cell line. This data indicates, as a consequence, that the levels of these known subunits are not responsible for the difference in GST enzymic activity found in PE04.

FIGURE 20 Glutathione S-transferase Subunit Content in
PE01 and PE04.

Western blots were carried out as described in the Materials and Methods (Section 2.6.0) using 50µg; A and C or 25µg; B of soluble protein per track. The bands were identified using the specific antibodies raised to the human Pi class (λ acidic) and alpha class (B₁ basic) subunits. STD = human GST standard.

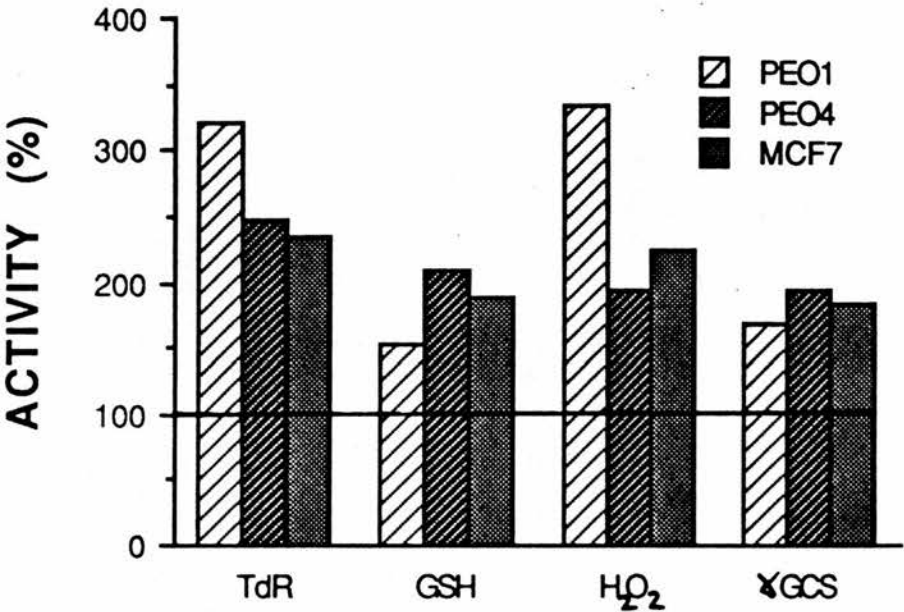


GSH levels have been shown to change during the cell cycle (Szent-Gyorgyi, 1978). Consequently, it was of interest to investigate which of the glutathione-dependent enzymes were also subject to such regulation, especially as they could contribute to the differences between the cell lines and also be a factor in their sensitivity to cytotoxic drugs.

Tritiated thymidine incorporation into both cell lines was measured to verify that the cells were either in confluency or in logarithmic growth. A two to three fold increase in tritiated thymidine incorporation in both cell lines, synchronised into logarithmic growth, compared with confluency, was found (Table 7, Figure 21). It was also shown, however, that there was a large difference in thymidine incorporation between PE01 and PE04, indicating a higher rate of DNA synthesis in PE04. In both PE01 and PE04, a significant elevation in reduced GSH levels was found in the lines during logarithmic growth (Table 7, Figure 21). The increase was 1.6 ($p < 0.01$) and 2.1 ($p < 0.001$) fold, relative to the confluent cultures for PE01 and PE04 respectively. In cultures in logarithmic growth, GSH content was higher in PE04 by 1.8 fold compared with PE01, the difference being slightly higher than that observed for confluent cultures. A significant increase in GSH levels (1.9 fold) was also observed for the breast cell line MCF7 during logarithmic growth. An increase from 34.2 ± 17.4 nmoles GSH/mg protein to 64.7 ± 17.0 nmoles GSH/mg protein. Concomitant with the increase in GSH levels an increase (1.4 to 2 fold) in γ GCS activity was found in all three cell lines (Table 7, Figure 21). The enzymatic activity increased 1.8 fold from 7.1 ± 2.3 to 13.0 ± 3.7 nmol/min/mg protein for the MCF7 cell line. The selenium-dependent GPX was also found to change in the cell lines in logarithmic growth. GPX activity, using hydrogen peroxide as a substrate, was elevated in dividing cells in all three cell lines, (Table 7, Figure 21) 1.9 to 3.4 fold, GPX activity being increased in MCF7 cell line from 34.1 ± 10.1 nmol GSH oxidised/min/mg protein to 76.8 ± 21.7 nmol GSH oxidised/min/mg/protein. Other glutathione-dependent

FIGURE 21 **Cell Cycle Effects on Glutathione-Dependent Enzyme Activity**

Logarithmic and confluent cell cultures of PEO1, PEO4 and MCF7 were harvested with trypsin:versene and prepared as described in the Materials and Methods (Section 2.2.0). All the assays were carried out on the soluble fraction. Activity of cells in logarithmic growth is expressed as a percentage of the confluent culture. Values are given in Table 6 and in the text of Section 3.2.2. The abbreviations used are indicated in legend to Table 6. Td^r represents ³H thymidine incorporation.



enzymes studied, however, GST, γ GT and GRD, were all unchanged during the cell cycle (Table 7).

(c) Human sarcoma cell line showing multidrug resistant phenotype.

GST activity was measured using CDNB in the wild type (MES-SA) and multidrug resistant (DX5) cells showed that DX5 cells had a 2.4 fold higher activity ($p < 0.001$) and a 1.3 fold higher GSH content than the wild type line (Table 8, figure 22). Assays involving model substrates, which give an indication of which GST isoenzyme subgroups were overexpressed in the resistant line, were also carried out. The activities towards cumene hydroperoxide and ethacrynic acid were unchanged in the resistant DX5 line, (Table 8), compared with the parental line MES-SA. Selenium-dependent glutathione peroxidase activity towards hydrogen peroxide was, in contrast, significantly elevated in DX5 (12.7 fold, $p < 0.001$). Consequently, total glutathione peroxidase activity, which was found unchanged between the lines, must be completely due to the non-selenium-dependent activity of alpha class GST's present in the MES-SA wild type line. Other glutathione-dependent enzyme activities i.e. γ GCS, GRD and γ GT, were determined in both of the cell lines, but only γ GT activity was found to be significantly elevated (6.7 fold, $p < 0.001$) in the drug resistant DX5 line.

As in the in vivo ovarian adenocarcinoma cell line model, Western blots were carried out to establish the GST subunit composition of the wild type MES-SA and the multidrug resistant DX5 cell lines, and also to determine whether the difference in GST activity towards CDNB and the activities towards the model substrates ethacrynic acid and cumene hydroperoxide could be associated with differences in the expression of known GST subunits (Figure 23). In both cell lines, high levels of the pi class (λ) GST was detected, using antibodies raised against the human pi class protein. Similarly, the mu class (μ) protein was found at the same levels in both MES-SA and DX5 cells, but at much lower levels. A difference, however, was observed in the alpha class

Table 8: Glutathione-dependent enzyme expression in MES-SA and DX5 cell lines.

Cell Line	GSH	GST	GPX				γ GT	γ GCS	GRD
			CDNB	EA	CHP	H ₂ O ₂			
MES-SA	11.0 \pm 20.1	66.4 \pm 14.1	9.4 \pm 1.8	5.4 \pm 0.8	0.33 \pm 0.01	0.56 \pm 0.06	279.6 \pm 30.2	173.0 \pm 20.6	
DX5	143.1 \pm 17.6	160.8 \pm 10.4*	8.2 \pm 1.2	4.2 \pm 0.3	4.2 \pm 0.3*	3.83 \pm 0.07*	249.8 \pm 31.4	120.0 \pm 30.4	

The abbreviations used are : GSH, glutathione; GST, glutathione S-transferase; GPX, glutathione peroxidase; γ GT, γ glutamyl transpeptidase; γ GCS, γ glutamyl cysteinyl synthetase; GRD, glutathione reductase; CDBN, 1-chloro2,4-dinitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide. All reactions were carried out at 37°C. Values expressed as nmoles/min/mg protein for CDBN, CHP, EA, H₂O₂, γ GCS and GRD or nmoles/min/10⁶ for γ GT. GSH content is expressed as nmol/mg protein.

* P < 0.001.

FIGURE 22 Relative Expression of Glutathione-Dependent
Enzymes in DX5 Cells.

The DX5 values shown are expressed as percentages of the MES-SA wild type line. See Table 7 for further details.

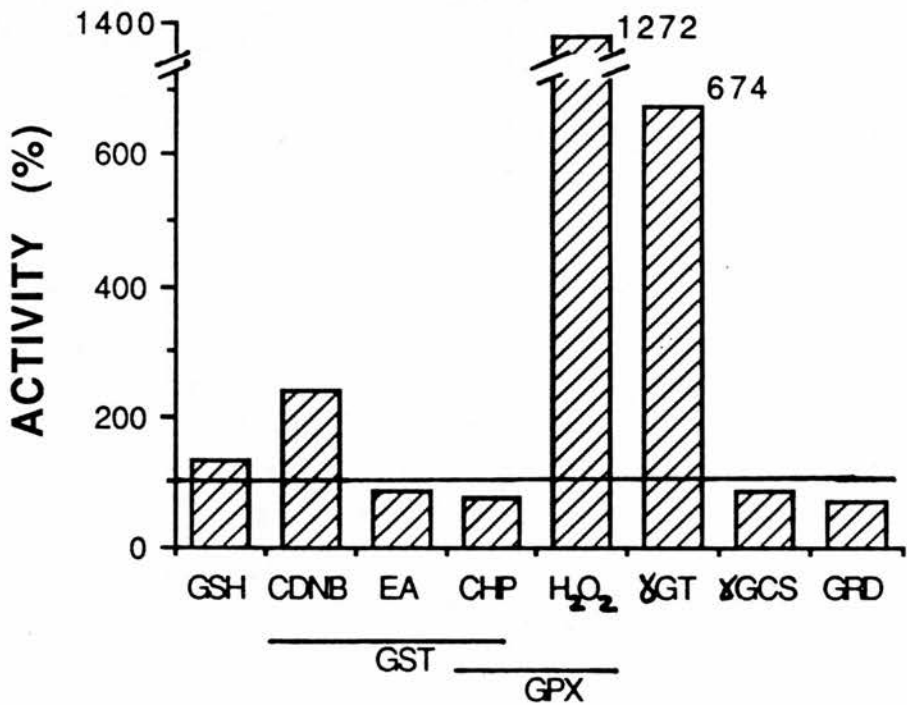
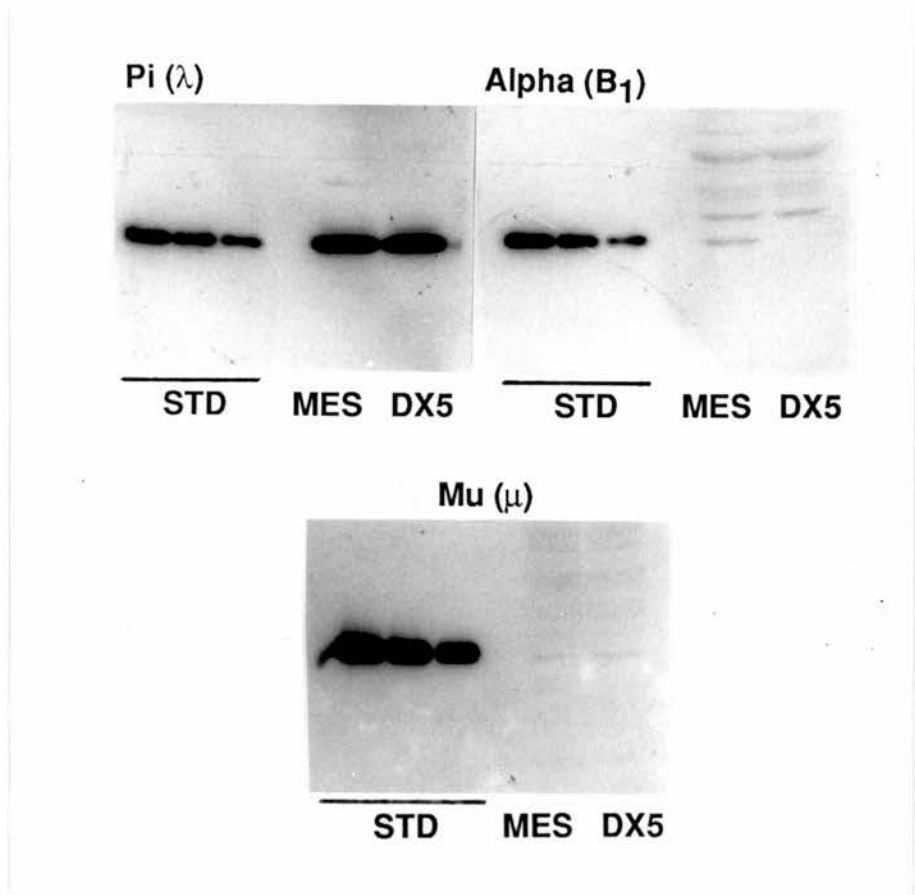


FIGURE 23 Glutathione S-transferase Subunit Content in MES-SA
and DX5

Western blots were carried out as described in the Materials and Methods (Section 2.6.0) using 50µg of soluble protein per track. The bands were identified using specific antibodies raised to the human pi class (λ), alpha class (B₁) and mu class (μ) subunits. STD represents human GST standard. MES and DX5 are the wild type and resistant cell lines respectively.



(B₁) GST expression, with the human alpha protein found in MES-SA and not in the resistant DX5 line. The Western blot data, therefore, confirmed that the enzyme increase in GPX activity was not as a result of increased expression of the alpha class GST subunits.

3.2.3 Discussion.

The elevation of GST's in drug resistant cell lines has been shown by several groups (Wang & Tew, 1985; McGowan & Fox, 1986; Batist, et al., 1986), but the identification of which GST subunits are overexpressed in these resistant lines has not been identified in all cases. Batist, et al., (1986) has shown that the pi class (λ) protein was elevated in his drug resistant MCF7 cell line, the same class of GST which has been shown to be elevated in experimental models such as preneoplasia (Kitahara, et al., 1984). In this study, however, where GSH and glutathione-dependent enzymes, such as the GST's, have been characterised in a CHO cell line made resistant to chlorambucil, it is the alpha class Yc GST subunit that was found to be overexpressed. This finding is in agreement with a study carried out by Buller, et al., (1987) who has preliminary evidence to show differences in the GST subunit composition between a wild type and chlorambucil resistant Walker 256 rat mammary carcinoma cell line. These differences included an increase in alpha class Yc subunit in the resistant line, although this was not conclusively proved. The involvement, therefore, of alpha class Yc subunit in the detoxification of chlorambucil, is likely. Further evidence for this possibility comes from studies which have shown that melphalan, which is a nitrogen mustard structurally similar to chlorambucil, is a substrate for the GST-catalysed conjugation with GSH (Dulik, et al., 1986). A further extension to this work would be to use the purified alpha Yc GST subunit to show directly whether chlorambucil is detoxified by this subunit to a GSH-chlorambucil conjugate. This would provide conclusive evidence of a direct role for the

action of alpha class Yc subunit in the resistance of the CHO-Chl^r cell line to nitrogen mustards.

The purified alpha class Yc subunit from the resistant CHO-Chl^r line, which is the first GST subunit to be purified from Chinese hamster, was only partly characterised due to limitations in time. It was, however, shown to be equivalent to the rat alpha class Yc subunit by Western blotting and model substrate analysis. Molecular weight of the purified alpha class Yc subunit was 27,500, which is slightly higher than the 25,000 molecular weight overexpressed protein found in the CHO-Chl^r resistant line by Robson, et al. (1986).

Specific activity values for model substrates for the purified alpha class Yc subunit were found to be lower than expected for other purified rat GST's (Mannervik, 1985). There may be several explanations for this; a) the CHO Yc protein may have lower turnover numbers for the substrates than the equivalent rat enzyme, or b) the S-hexyl glutathione used to elute the purified protein from the S-hexyl glutathione affinity column may not have been completely removed, and therefore may have inhibited the enzyme during the activity measurements. In support of the second possibility, purification of rat and hamster liver GST's, using S-hexyl glutathione to elute the proteins from the S-hexyl glutathione affinity column, results in lower CDNB activity than if the subunits were purified using a glutathione affinity column (Hayes, personal communication). The ratio of the model substrate activities for cumene hydroperoxide and ethacrynic acid to CDNB activity, are similar to alpha class rat GST's (Mannervik, 1985). The identity of the purified GST subunit could have been verified by NH₂-terminal sequence analysis also. Inhibitor studies using inhibitors which tend to be specific for certain GST subunits (Table 4) could also substantiate whether the purified protein was indeed an alpha class Yc subunit.

It would have been of interest to establish whether a low dose of a selective agent, in this case chlorambucil, to the wild type CHO cells would have induced a transient increase in the alpha class Yc subunit expression, and whether this increase in GST subunit concentration could protect the cell line against a higher, more toxic, dose of chlorambucil. This type of experiment has been carried out in normal bone marrow cells, with results described in Section 4.1.0.

Cloning of the gene coding for this over expressed alpha Yc GST subunit would also have provided important information relating to the regulation of expression of the GST's in drug resistance, and also whether this protein can confer resistance towards chlorambucil. The over expression of the Yc GST subunit was, in fact stable, in that the cells did not need to be cultured in the presence of chlorambucil.

Apart from the increase in alpha class Yc subunit in the resistant cell line, an increase in reduced GSH was also found. This elevation in GSH levels is consistent with other reports where elevated GSH levels has been reported in a melphalan-resistant L1210 cell line (Suzukake, et al, 1983). The elevation in GSH was also associated with increased melphalan breakdown. In the chlorambucil resistant CHO-Chl^r cell line, therefore, it appears that the resistance is due to a combination of increased GSH, alpha class Yc GST subunit and γ GT levels.

Similar changes were also observed in the ovarian cell lines obtained before, and after, a patient became resistant to chemotherapy. In this model, other glutathione-dependent enzyme changes were also observed.

The two ovarian cell lines used in this study represent a valuable in vivo model for the investigation of drug resistance. Karyotype analysis showed that the cell lines were of the same clonal origin, but they diverged from each other either before or during drug treatment (Wolf et al, 1987b). Although it is not clear how representative the cell lines are of the original tumour, the

information in this study provides evidence that the tumour cells isolated from a patient who had become resistant to drug therapy contained higher levels of reduced GSH, and a variety of glutathione-dependent enzymes, than those obtained from the same individual prior to the onset of drug resistance. It is important to remember, however, that the limitation in using cell lines is that phenotypic changes may occur in the cells during the transition into culture.

Cis platinum resistant PE04 cell line was found to have elevated selenium-dependent GPX activity, an enzyme which has been shown to be involved in the protection of cells from cytotoxic chemicals (Smith, et al. 1983), and, in particular, in the reduction of organic and lipid hydroperoxides generated from such compounds as anticancer drugs (eg. adriamycin). Interestingly, the cis platinum-resistant PE04 cell line was also found to be cross resistant to adriamycin (Hayward, PhD Thesis 1987, Edinburgh), unlike the CHO cell line CHO-Chl^r which was sensitive to adriamycin. The increase in selenium-dependent GPX activity may, therefore, have a direct role in the cis platinum resistant PE04 cell line, in determining the cross resistance to adriamycin.

There is a growing body of evidence which indicates that reduced GSH content may be an important factor in determining the susceptibility of tumour cells to a wide range of distinct cytotoxic drugs. In regard to the resistance of cells to cis platinum, there are conflicting reports as to the relationship between drug susceptibility and GSH levels (Andrews, et al. 1985; 1986; Louie, et al. 1985) (see also Section 1.11.0). In the study described here, there was an approximate two fold increase in reduced GSH levels in the cis platinum resistant PE04 cell line, compared with the sensitive PE01 line, in agreement with the study of Louie et al. (1985). There is also strong evidence to show that GSH plays an important role in the protection against alkylating agents. This is particularly the case for ovarian cancer where Ozols and Co-workers have demonstrated that GSH depletion will sensitise cells to

the toxic effects of melphalan, cis platinum and adriamycin (Green, et al. 1984; Hamilton, et al. 1985).

A substantial difference between the cell lines was observed in GST activity, which was not directly attributable to expression of any of the isoenzymes studied. Whether novel forms of these enzymes are present in ovarian tissue, or whether Western blots did not reflect the active enzyme concentration, is at present unclear. A further experiment which could have been carried out to clarify the situation, would have been to use antibodies raised against the three known human GST subunit classes to immunoprecipitate the respective proteins, and look to see whether this will abolish the differences between the lines. There is, however, evidence in reports, indicating that the expression of GST's within tumours is an important factor in determining their sensitivity to anticancer drugs such as melphalan (see Section 1.11.0) (Hayes & Wolf, 1988; Dulik, et al. 1986), cyclophosphamide (Adams, et al. 1985; McGowan & Fox, 1986) and also, as indicated above, chlorambucil (Robson, et al. 1987). In addition, a human squamous carcinoma cell line, resistant to cis platinum, had a 3 fold higher GST content than the sensitive line. However, the GST subunit involved was not identified (Teicher, et al. 1987). Higher GST levels in the cis platinum resistant PE04 cell line may, therefore, contribute to the differences in drug sensitivity observed. In this regard, it is interesting that both cell lines contained relatively high levels of the pi class subunit (λ) which has often been discussed in relation to the resistance of cells to toxic compounds (Kitahara, et al. 1984; Batist et al. 1986; Hayes & Wolf, 1988). It would have been interesting to have made the sensitive PE01 cell line resistant to cis platinum, to determine whether the same changes in GSH and glutathione-dependent enzymes, such as γ GT, selenium-dependent GPX and GST occur, as was observed in the PE04 cell line.

GSH concentration, and the activity of γ GCS and selenium-dependent GPX, were elevated in dividing cells. These changes were observed in both the

ovarian lines and a MCF7 cell line. In general, PE01 and PE04 cell lines were, however, affected in a similar manner, although the extent of the cell cycle effects were slightly different. This may play a role in the relative sensitivity of the cell lines to cytotoxic drugs; however, the effect was not sufficiently pronounced to make a clear conclusion. Cell cycle differences in the effects of cytotoxic drugs and carcinogens are well documented (Frei & Harsano, 1967; Watanabe & Horikawa, 1975), and the over expression of GSH could explain the increased resistance of dividing cells to certain carcinogens (Dunn, 1972; Fohlmeister et al. 1984). The increase in GSH concentration found in the cell cycle may be due to the direct consequence of the increase in rate determining activity of γ GCS. The cell cycle effects observed in this study, exemplify the need for carefully controlled conditions when measuring, and comparing, GSH and related enzymes in cell culture.

The changes which occurred in the multidrug resistant DX5 cell line were similar to that of the ovarian PE04 cell line, with increases in GSH, γ GT and selenium-dependent GPX activity. The MDR cell line was initially made resistant to adriamycin; therefore, one of the possible defence mechanisms against this drug, apart from the over expression of GP180, could be the increase in selenium-dependent GPX activity. As described for the PE04 model, there was also higher GST activity towards CDNB, detected in the MDR DX5 cell line, compared with the wild type MES-SA line, which could not be accounted for by an increase in expression of any of the GST isoenzymes studied by Western blotting or model substrate analysis. The purification of these GST's would have been desirable, but not possible, with the time available. Interestingly, it was observed by Western blot analysis that a human alpha class (B_1) protein was detected in the wild type MES-SA line which was not present in the MDR DX5 line. One explanation for this could be that selenium metabolism is different in the lines, as it has been well described that selenium

levels can modulate both alpha class GST levels and selenium-dependent GPX levels (Masukawa et al, 1983).

Evidence from this study indicates that, as well as the DX5 line exhibiting characteristic MDR phenotype, and the over expression of GP180, this particular cell line also has increased levels of GSH and glutathione-dependent enzymes, including the GST's. In this report, it agrees with Batist et al (1986) study, in which an adriamycin resistant MCF7 cell line had higher levels of glutathione peroxidase, and GST activity. The GST involved, however, was suggested to be a novel pi class GST, although this was not clearly demonstrated. This may allow the postulation of the theory of possible co-ordinate regulation of drug induced detoxification systems, such as GP180 and the GST's, as Thorgeirsson, et al (1987) has shown the elevation of GP180 in liver preneoplastic foci and regenerating liver, two models in which GST's have also been shown to be elevated. In order to substantiate this theory, a lot more experimental work would have to be carried out, including looking at more MDR cell lines for changes in GSH and glutathione-dependent enzyme levels.

Similar changes in GSH and glutathione-dependent enzymes were found in both the in vivo and in vitro models of drug resistance as discussed above; however, it is important to note that the levels of drug resistance were higher in the in vitro model compared with in vivo. In vivo drug resistance, of which this is the first study carried out, only requires a small increase in the resistance of a tumour to an anticancer drug (1.5 - 3 fold) to be of clinical significance, as the dose currently used is already at the limit of tolerated toxicity. A remarkable similarity, however, in the phenotypic changes in glutathione-dependent enzymes in the cells made resistant in vivo or in vitro to cytotoxic drugs, was observed. The molecular mechanisms of these changes in protein levels, found in all three models, are not clear. It is possible that various cells which over express these proteins are selected during the

generation of these lines, or that phenotypic changes occur during prolonged exposure to that cytotoxic agent ie. an adaptive response (see later).

In this study, however, the most consistent inductions in all three models were GST's, and in particular GSH and γ GT levels. The elevation in GSH levels found in the resistant lines may be a direct consequence of large increases in γ GT activity, as this enzyme has been implicated in the regulation of cellular levels of this co-factor (Griffith & Meister, 1979c; Meister, 1983; Ahmad, et al. 1987). Recently, it has been determined in mouse lymphoma L1210 cells, made resistant to L-phenylalanine mustard (L-PAM), that there was a close correlation between γ GT levels and levels of GSH observed (Ahmad, et al. 1987); this data is consistent with the findings here.

As indicated above, GSH levels were also found to be elevated in all the drug resistant cell line models studied; this is consistent with other work in the literature which has indicated, for example, that in L1210 cells resistant to melphalan, there is an elevation in reduced GSH levels. In a human ovarian cancer cell line, however, where elevated GSH levels were shown, the cells could be resensitised by using BSO (Green, et al. 1984; Hamilton, et al. 1985). Use of BSO to deplete GSH levels in CHO-Chl^r (Robson, et al. 1987) did not sensitise the cells to any great extent. This indicates, therefore, that GSH may play only a small, direct, role in the resistance mechanism. It is more likely that GSH is involved in the resistance mechanism by acting as a cofactor for other glutathione-dependent enzymes which are involved in the detoxification process.

Selenium-dependent GPX activity was found to be increased in both DX5 and PE04, both cell lines which showed resistance to adriamycin, an anticancer drug which is thought to act through generation of superoxide radicals. Interestingly, however, in these lines the alpha class GST's, which also have GPX activity, were observed to be either decreased, or unchanged, as in the PE04 cell line. In contrast, however, in the CHO-Chl^r cell line selenium-

dependent GPX, activity was found to be unchanged, with a dramatic increase in the alpha class Yc GST subunit. There is, therefore, a possibility that there could be coordinate inverse regulation between selenium-dependent, and non-selenium-dependent GPX (alpha GST activity) activity in the different cell lines. Selenium levels, as mentioned previously, can affect the differential expression of the two types of GPX activity; it is, therefore, possible to speculate that different cytotoxic drugs may influence either the regulation of expression of the GPX activities, or affect selenium levels, which, in turn, may influence the expression of the two types of GPX activities.

In conclusion, evidence was presented demonstrating higher levels of expression of GSH and glutathione-dependent enzymes in all three cell line models, following the acquisition of resistance to cytotoxic drugs. It is of importance to note that a variety of glutathione-dependent enzymes, in addition to GST, were also elevated in the drug resistant lines, and these may also play an important role in drug resistance. These enzymes may be elevated in an adaptive response to the cytotoxic drugs used.

Chapter 4.0.0 **GLUTATHIONE-DEPENDENT ENZYMES IN ADAPTIVE RESPONSE TO CHEMICALS.**

4.1.0 Chemotherapeutic drug induced resistance in normal bone marrow cells.

4.1.1 Introduction.

The use of cytotoxic agents in cancer chemotherapy is often limited by the action of these agents on the normal homeostatic proliferating cells of the body, particularly those of the bone marrow. It has been known for many years, however, that exposure of animals to a low dose of a cytotoxic compound, such as anticancer drugs, have the ability to protect against a normal lethal dose of the same, or in some cases structurally different, cytotoxins (Miller, et al, 1975; Kimball, et al, 1976; Boyd, 1980). These so called 'priming' effects are found to be independent of tissue and have been described for a variety of toxins which are specific for the lung, gut and bone marrow. In particular, cyclophosphamide (CP), a chemotherapeutic agent which causes myelotoxicity, has recently been studied in this regard. This effect represents an adaptive response to chemicals which results in resistance to a subsequent normally lethal dose.

Characterisation of these changes, in GSH and total GST levels in mouse bone marrow following a 'priming' dose of CP, has been carried out by Adams et al, (1985). These workers have shown an elevation of GST (2-3 fold) and GSH (2-3 fold) occurs exactly at the same point as maximum protection against a lethal dose of the same compound, that is on day 5 and day 6 after the 'priming' dose. This change was observed to be localised within the granulocyte population (Carmichael, et al, 1986).

In the study described here, the specific GST subunit which was involved in the resistance mechanism of the 'priming' phenomenon was determined, and also whether glutathione-dependent enzymes such as γ GT, γ GCS, GRD and GPX are also elevated.

4.1.2 Results.

GST activity using CDNB as a substrate was determined in the soluble fraction of bone marrow cells taken from 'primed' and 'unprimed' (control) mice on days 5 and 6, i.e. on the days of maximal protection against CP (Adams et al., 1985). As indicated in Table 9, Figure 24, GST activity was significantly elevated (2.9 fold $p < 0.002$) on both days in the 'primed' samples. In addition, the peroxidase activity towards cumene hydroperoxide was also elevated (1.5, $p < 0.007$; 1.8 fold $p < 0.01$ for days 5,6). The increased glutathione peroxidase activity appeared to be due to differences in the expression of the GST as the selenium-dependent activity (determined using H_2O_2 as a substrate) was similar in both 'primed' and control bone marrow samples. Pi class GST activity towards ethacrynic acid was found to be unchanged in both the samples, as were the other glutathione-dependent enzymes studied.

The change in GST activity appeared to be due to an elevation in the alpha class and mu class (Yb) subunits. The protein staining pattern of soluble fraction clearly showed a significant elevation in the primed samples of a protein with a mobility similar to that of the rat Yb GST subunit and a slight elevation in an alpha GST subunit in the primed samples on both days (Figure 25). Western blotting using specific antisera raised against human alpha (B_1), mu (μ), and pi (λ) GST proteins showed a 2 fold increase in the level of a mu GST subunit, as determined by densitometer scanning, and also increase in a protein equivalent to rat alpha (Ya), in the 'primed' samples. Levels of the alpha class GST were not seen in the control samples, but relatively high levels of the pi and mu class GST proteins could be observed (Figure 26).

Carmichael et al., (1986) indicated that the differences observed in 'priming' were confined to the granulocyte population. As a consequence, peripheral granulocytes and peripheral lymphocytes were prepared from

Table 9: Glutathione-dependent enzyme expression in bone marrow Day 5 and Day 6 from 'primed' and control mice.

		Activity						
		GST		GPX		γ GT	γ GCS	GRD
		CDNB	EA	CHP	H ₂ O ₂			
Day 5	C	28.8±4.5	9.3±1.8	48.4±2.3	49.6±2.4	0.23±0.03	25.8±3.7	136±6
	P	84.7±12.6 *	7.9±2.8	72.7±7.8+	50.0±10.4	0.21±0.03	23.0±1.7	135±4
Day 6	C	26.4±3.4	10.0±0.6	45.8±4.4	54.4±4.8	0.21±0.02	23.9±2.5	142±6
	P	76.2±5.9**	9.3±2.6	82.4±4.6**	57.3±2.1	0.21±0.006	22.7±3.2	143±5

The abbreviations used are the same as for Table 7. C is control, P is primed. All reactions were carried out at 37°C. Values expressed a nmoles/min/mg protein for CDNB, CHP, EA, H₂O₂, γGCS and GRD or nmoles/min/10⁶ cells for γGT.

* p<0.002; **p<0.001; +p<0.007

FIGURE 24 Relative Expression of Glutathione-Dependent Enzymes
In (A) Bone Marrow. (B) Peripheral Granulocyte and (C)
Peripheral Lymphocyte Cells taken from 'Primed' Mice
on Days 5 and 6.

The values shown are expressed as percentages of the control 'unprimed' mice. For abbreviations see Table 7.

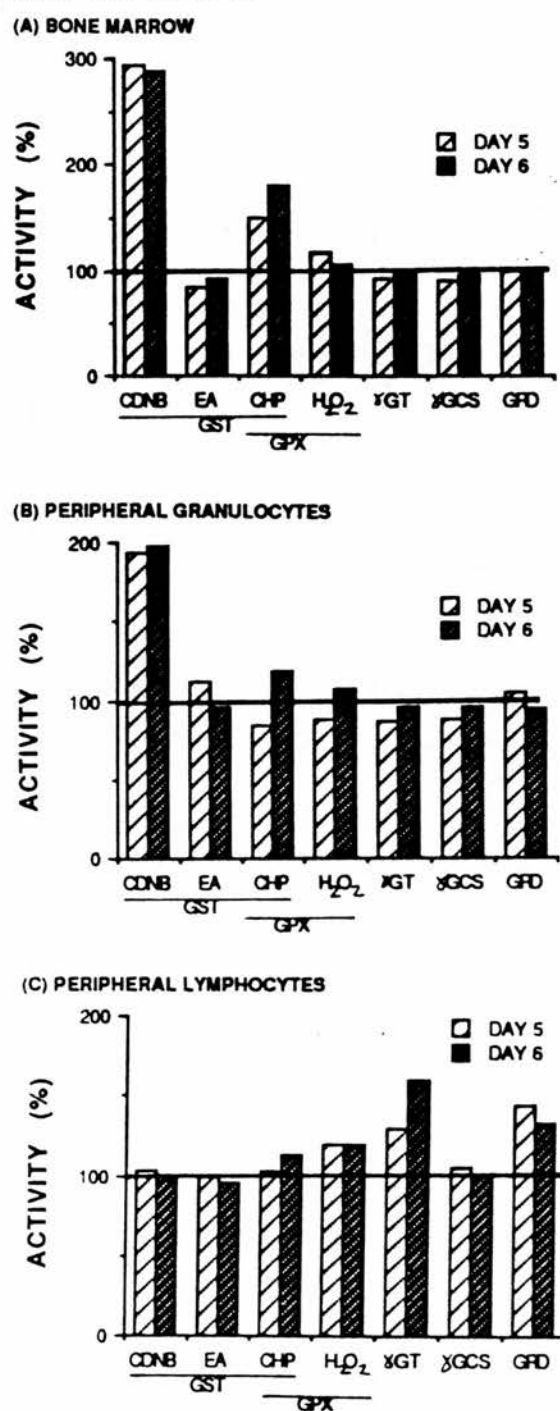
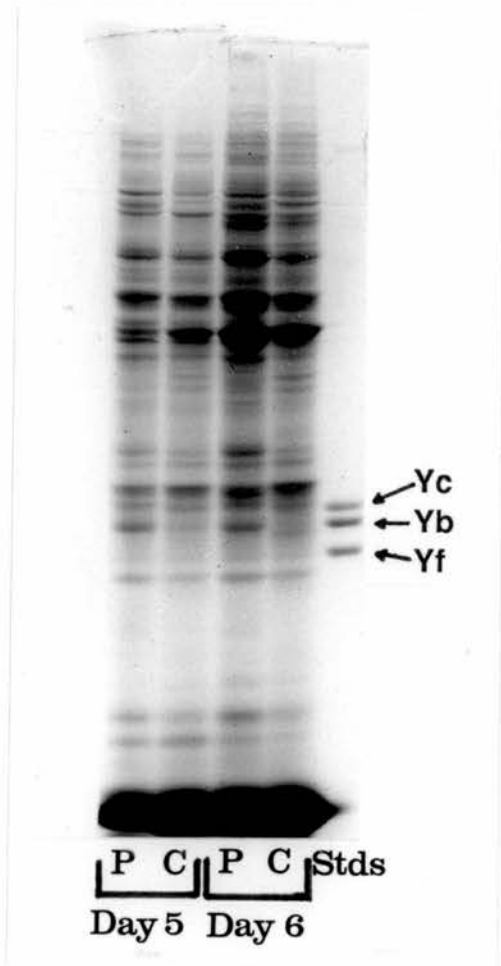


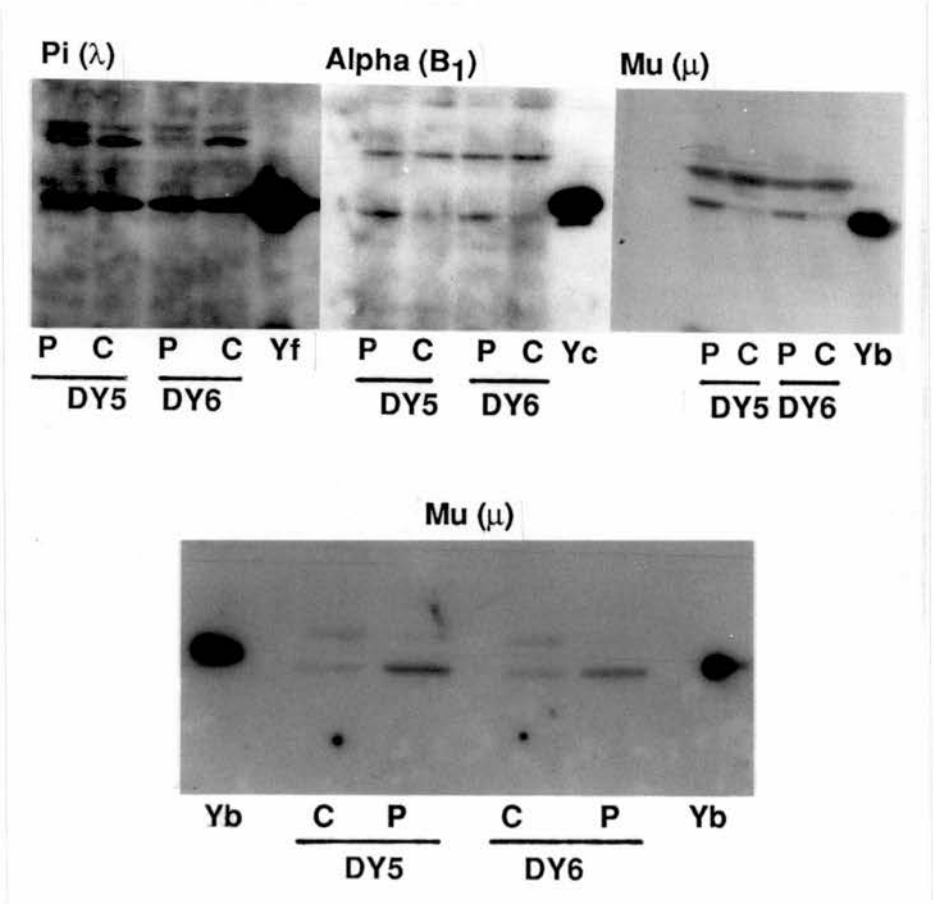
FIGURE 25 Protein Staining Pattern of 'Primed' and Control Bone Marrow.

Samples were separated by SDS-PAGE. 100µg of soluble protein was loaded per track. The mobilities of the rat Yc, Yb and Yf standards are also shown. P and C represent the primed and control samples respectively, on days 5 and 6.



**FIGURE 26 Glutathione S-transferase Subunit Content in 'Primed'
and Control Bone Marrow.**

Western blots were carried out as described in the Materials and Methods (Section 2.6.0) using 100µg of soluble protein per track. The bands were identified using specific antibodies raised to the human pi class (λ), alpha class (B_1) and mu class (μ) GST subunits. Standards used were rat Yf, Yc and Yb₁ GST subunits. P and C were 'primed' and control samples respectively on days 5 and 6.



'primed' and control mice, on which GST and glutathione-dependent enzyme assays were determined. CDNB was 2 fold higher ($p < 0.006$, $p < 0.003$ Day 5, 6 respectively) in the peripheral granulocyte and unchanged in the peripheral lymphocyte population of cells from the 'primed' mice on days 5 and 6 (Tables 10, 11 and Figure 24). Glutathione peroxidase activities in these samples towards cumene hydroperoxide were unchanged. Other glutathione-dependent enzymes were also found to be unchanged in granulocytes, although an elevation in both γ GT (1.3, $p < 0.02$; 1.6 $p < 0.01$ for days 5,6) and GRD (1.5, $p < 0.001$; 1.3 $p < 0.05$ for days 5,6) was found in the peripheral lymphocytes (Tables 10, 11 and Figure 24).

Western blots using the antibodies against the human alpha, mu and pi class GST proteins, indicated changes in GST subunit expression not only between 'primed' and control peripheral granulocytes, but also between peripheral granulocyte and peripheral lymphocyte cells (Figure 27). On day 5 and 6 following priming, there was an increase in the levels of Yb protein, and also a faint increase in Ya GST subunits in the peripheral granulocytes. No change in the pi class GST was detected. In contrast, no changes in GST subunits was observed in the peripheral lymphocytes. Interestingly, there were differences in GST subunit expression between the peripheral granulocyte and lymphocytes (Figure 27). Both cell types contained mu GST's, but the peripheral lymphocytes contained high levels of the pi protein. The converse was observed, however, for the alpha GST subunit expression.

As indicated, differences in GST subunit expression were observed in peripheral granulocytes and peripheral lymphocytes (Figure 27). Populations of these cell types were sorted on a fluorescence activated cell sorter (FACS), in conjunction with Dr. J. Ansell, Dept. Zoology, University of Edinburgh, from the 'primed' and control mouse bone marrow cells. Unfortunately, not enough cells were sorted, in over eight hours continuous sorting, to provide enough

Table 10: Glutathione-dependent enzyme expression in peripheral granulocytes Day 5, Day 6 from 'primed' and control mice.

		Activity						
		GST		GPX		γ GT	γ GCS	GRD
		CDNB	EA	CHP	H ₂ O ₂			
Day 5	C	13.9 \pm 1.4	2.8 \pm 0.4	54.1 \pm 10.8	48.1 \pm 7.1	0.73 \pm 0.14	54.8 \pm 3.0	138 \pm 6
	P	26.9 \pm 4.0*	2.8 \pm 1.1	45.2 \pm 6.0	42.3 \pm 3.2	0.63 \pm 0.04	48.0 \pm 10.6	145 \pm 4
Day 6	C	14.2 \pm 2.5	2.3 \pm 0.4	47.0 \pm 3.8	44.1 \pm 3.8	0.67 \pm 0.04	53.3 \pm 6.8	142 \pm 7
	P	28.0 \pm 2.5**	2.2 \pm 0.1	53.8 \pm 5.8	47.2 \pm 1.01	0.64 \pm 0.06	50.5 \pm 2.5	134 \pm 6

The abbreviations used are the same as for Table 7. C is control, P is primed samples. All reactions were carried out at 37°C. Values expressed as nmols/min/mg protein for CDNB, CHP, EA, H₂O₂, γ GCS and GRD or nmols/min/10⁶ cells for γ GT.

* p<0.006; **p<0.003 .

Table 11: Glutathione-dependent enzyme expression in peripheral lymphocytes Day 5, Day 6 from 'primed' and control mice.

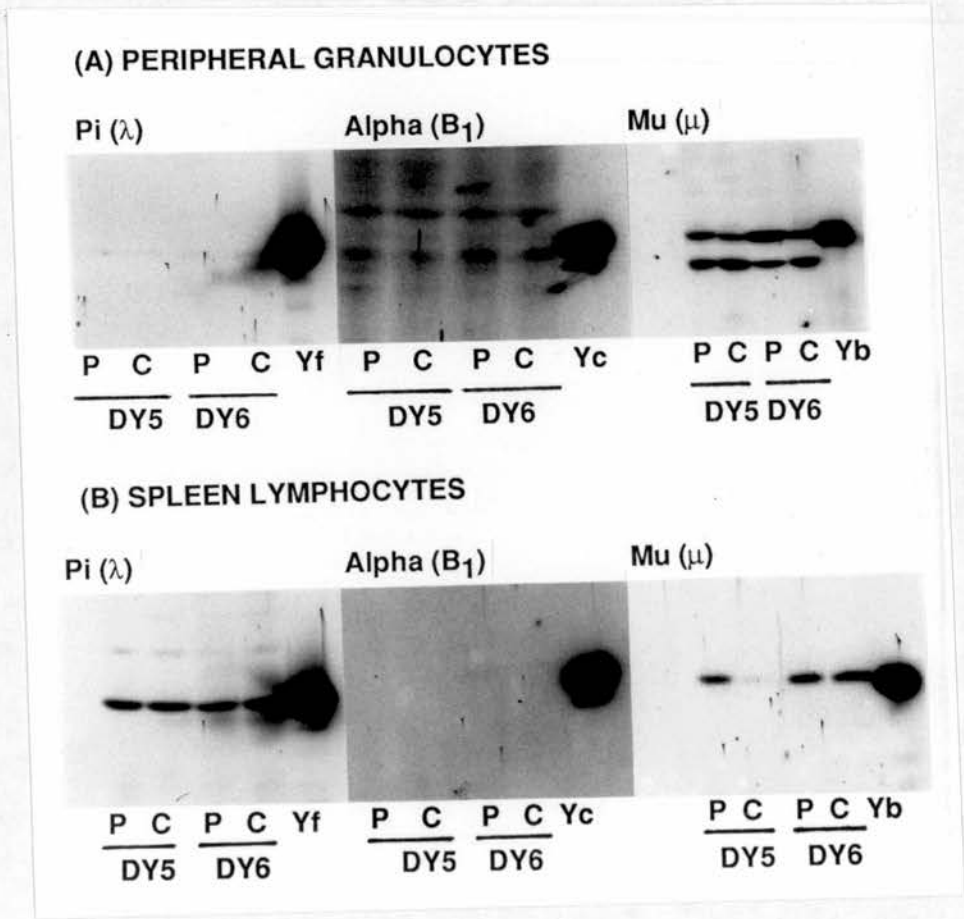
		Activity						
		GST	GPX		γ GT	γ GCS	GRD	
		CDNB	EA	CHP	H ₂ O ₂			
Day 5	C	52.6 \pm 5.7	4.2 \pm 1.7	41.1 \pm 12.9	41.9 \pm 1.2	0.6 \pm 0.03	54.9 \pm 3.7	153 \pm 9
	P	54.0 \pm 3.6	4.7 \pm 0.4	42.3 \pm 5.0	50.2 \pm 1.2	*0.80 \pm 0.03	58.5 \pm 4.4	+222 \pm 5
Day 6	C	54.4 \pm 5.4	4.3 \pm 0.6	39.4 \pm 2.5	41.5 \pm 3.3	0.47 \pm 0.07	56.9 \pm 2.8	187 \pm 4
	P	52.6 \pm 3.3	4.1 \pm 1.0	45.0 \pm 1.5	49.5 \pm 1.5	**0.75 \pm 0.08	58.2 \pm 1.5	++250 \pm 3.1

The abbreviations used are the same as for Table 7. C is control, P is primed samples. All reactions were carried out at 37°C. Values expressed as nmoles/min/mg protein for CDBN, CHP, EA, H₂O₂, γ GCS and GRD or nmoles/min/10⁶ cells for γ GT.

* p<0.002; **p<0.01; +p<0.001; ++p<0.05.

**FIGURE 27 Glutathione S-transferase Subunit Content In 'Primed'
and Control (A). Peripheral Granulocytes and (B)
Peripheral Lymphocytes.**

Western blots were carried out as described in Materials and Methods (Section 2.6.0) using 100µg of soluble protein per track. The bands were identified using specific antibodies raised to the human pi class (λ), alpha class (B_1) and mu class (μ) GST subunits. Standards used were rat Yf, Yc and Yb₁ GST subunits. P and C were 'primed' and control samples respectively.



sample to detect GST activity. Bone marrow cells also contain a population of erythrocytes. It was not possible in this study, however, to look at the GST subunit composition in peripheral erythrocytes taken from the 'primed' and control mice, due to the presence of large amounts of haemoglobin, which affects the amount of protein loaded.

4.1.3 Discussion.

GSH has been shown to be involved in the deactivation of 4-hydroxy cyclophosphamide (4-OH-CP) and acrolein (see Section 1.11.0), both metabolites of CP (see Figure 7). Acrolein is highly toxic, and is most probably the cause of the myelotoxic side effects of CP, including granulocytopenia. Higher levels of GSH and glutathione-dependent enzymes would, therefore, play an important role in protecting the cells of the bone marrow against the toxic side effects of CP.

The cross reactivity of the mouse GST subunits, with the antisera raised against the human GST isoenzymes, has been determined by Adams *et al* (1986) and McLellan & Hayes (1987), who found alpha class (Ya), mu class (Yb) and pi class (Yf) subunits in mouse liver. In the study described here, alpha, mu and pi class GST subunits were also detected in mouse bone marrow using these antibodies to the human GST subunits. Peripheral lymphocytes had high amounts of pi class (Yf) and mu (Yb₁), but no alpha class GST's. This GST subunit composition is similar to the finding of Seidegard *et al*, (1985), who identified mu subunits in human lymphocytes. The presence of pi class GST, also in human lymphocytes (not shown), is as suggested by Seidegard *et al* (1987). In confirmation of the report by Adams *et al* (1986) a 2 fold elevation of GST activity was found on days 5 and 6, following CP administration. Differences in the levels of these GST subunits would, therefore, influence the response of the cells to the cytotoxic agent used. In response to a low dose of the alkylating agent CP, an increase in mu (Yb) and alpha (Ya) GST subunits

were observed. Whether this change in GST subunit profile is involved in the increase in the resistance of bone marrow to a high dose of the same chemotherapeutic agent is unclear. However, GST's are implicated in the detoxification of nitrogen mustards (see Section 1.11.0). These changes in GST subunit composition were also found in the peripheral granulocyte population, in contrast to peripheral lymphocytes, where no change in GST profile was observed. This evidence is consistent with the finding that the granulocyte population of the bone marrow is resistant to the higher lethal dose of CP (Carmichael, et al, 1986). Interestingly, the only cell population in which glutathione-dependent enzymes, apart from GST's, were found to be changed was in the lymphocyte population. It may be possible, therefore, that the increases in γ GT and GRD involved in the maintenance of GSH levels found in this population were related to the defence mechanism of the lymphocytes to the chemotherapeutic drug.

The bone marrow is a mixture of many different cell types. It is a possibility that changes in the proportions of these cell types may, in fact, influence the changes in glutathione-dependent enzymes observed in the bone marrow. Carmichael et al, (1986) using a fluorescence activated cell sorter (FACS), separated out the different populations of cells from the bone marrow in primed and control mice and found that the erythrocyte population remained unchanged, while a marked loss of lymphocytes and an increase in the granulocyte population was determined. This change in the granulocyte population profile (Figure 8), and more importantly, the changes in GST subunits within the granulocyte cells, would probably be involved in resistance of the bone marrow cells to the myelotoxic effects of CP.

Further FACS experiments to sort out bone marrow cell populations would have confirmed the changes observed in this study. It is also possible that changes occurred in the erythrocytes. However, in order to study this it

would have been necessary to remove the haemoglobin from the erythrocyte samples, to see whether differences in GST subunit profile of these cells existed, and therefore be involved in the resistance to the chemotherapeutic agent. It is, however, very unlikely to be the case, as it is known that erythrocytes contain mainly pi class GST subunits (Marcus, et al, 1978) and in the study described here the pi class GST was unchanged in the bone marrow and in both lymphocytes and granulocyte cells on priming.

It is noteworthy that the overexpression of the GST subunit in the bone marrow are transient, in that the protection afforded to the myleotoxic effect of CP is also transient, with maximal resistance to the lethal dose of CP occurring on days 5 and 6 after the low 'priming' dose. This transient increase in GST subunits is different from other drug resistance models such as preneoplasia (see Section 4.3.0) and acquired drug resistance in cell lines (Section 3.2.0) where the elevation in GST subunits are immortalised. Perhaps this indicates a different mechanism of induction and regulation of expression in these models, compared with the chemotherapeutic drug induced resistance in normal cells.

Similar changes to those described for the chemotherapeutic agent CP have also been found for other cytotoxic drugs, such as arabinofuranosyl-cytosine (ara-c) and myleotoxic doses of radiation (Adams, et al, 1985). In addition, it has been shown in other cell types e.g. hepatocytes, that a low dose of carbon tetrachloride can protect against a normally toxic dose of this hepatotoxin, and the elevated expression of GST is also observed (Di Simplicio, 1982), although the GST subunits were not identified. This may indicate, therefore, that the transient elevation of GST subunits, as described here, to a low dose of a cytotoxic compound, may form part of a general adaptive response which confers resistance to further cytotoxic insults. This type of adaptive response is of clinical value, in that the myleotoxicity of certain

chemotherapeutic agents is overcome by giving an initial 'priming' dose of the compound to protect the bone marrow. The method of protection is unknown, but this data may provide an explanation for the phenomenon. Unfortunately, it is not known whether such low doses of anticancer agents may also 'prime' tumours in a similar manner, and, therefore, protect the tumour from the cytotoxic drugs being used.

4.2.0 **Adaptive Response to Oxygen**

4.2.1 Introduction.

Oxygen-derived toxic molecules like $O_2^{\cdot -}$, OH^{\cdot} , and H_2O_2 are continuously produced in cells. Their steady-state concentration is currently unknown, due to technical problems in their measurement (Chance *et al.*, 1979). These free radicals are chemically unstable and very reactive. They attack unsaturated double bonds in DNA, proteins, and especially polyunsaturated lipids, which are sensitive to peroxidation (Tappel, 1978). The production of these toxic molecules stems from the auto-oxidation of a variety of biological reactions, such as hydroquinones, catecholamines, and ferredoxin, etc; the activity of oxidase enzymes, such as xanthine or aldehyde oxidase; the electron transfer on the inner mitochondrial membrane (Turrens & Boveris, 1980); the cytochrome P-450-dependent system; and also from external agents such as radiation and a wide variety of foreign chemicals, including anticancer drugs such as anthracyclines. Anthracyclines, such as adriamycin, can be enzymatically activated to free radical semiquinones that either react directly with biological targets, such as DNA and RNA, or generate cytotoxic oxygen-dependent free radicals such as superoxide anions or hydroxyl radicals (Bachur *et al.*, 1978; Sinah *et al.*, 1984; see also Section 1.11.0). In order to protect against the intermediates generated as a result of aerobic metabolism and cytotoxic stress, various defense mechanisms have evolved. The enzymes

superoxide dismutase, catalase and glutathione peroxidase (GPX) appear central in this protection mechanism.

In a study similar to that described in section 4.1.0, Kimball et al. (1976) have shown that rat lung tissues exposed to hyperoxia, caused an induction in lung 'antioxidant' defences, which were important in decreasing the susceptibility of the lung tissue to a higher dose of toxic oxygen. In particular, it was shown that reduced GSH, superoxide dismutase, glucose 6-phosphate dehydrogenase, and the glutathione-dependent enzymes GRD and GPX were elevated in response to hyperoxic conditions.

In this study, carried out in collaboration with Mr. I. Wyatt, I.C.I. plc, Macclesfield, rats were exposed to similar hyperoxic conditions, as described by Kimball et al (1976), in order to look at the role of reduced GSH and other glutathione-dependent enzymes, such as γ GT, γ GCS as well as GRD and GPX, in the adaptive response to high oxygen conditions in both lung and liver tissues. In particular, GST activities were also determined in order to establish whether they were also part of this adaptive response to oxidative stress. In this regard, an involvement of the GST's may be expected.

4.2.4 Results.

GSH and glutathione-dependent enzyme expression in high oxygen and control treated rat lung and liver tissues is shown in Table 12 and Figure 28. Using CDNB as a substrate, no difference in GST activity was detected in the hyperoxic treated livers, while there was a slight ^{but} significant ($p < 0.05$) decrease in GST activity in the hyperoxic lung samples. The peroxidase activity towards cumene hydroperoxide was significantly elevated (1.5 fold, $p < 0.05$) in the high oxygen treated lung samples, with no difference seen in the liver tissues. The increased GPX activity could be accounted for by an increase in the selenium-dependent GPX enzyme, since a 1.6 fold ($p < 0.005$) elevation in activity towards H_2O_2 was measured. Alpha class GST's cannot metabolise this

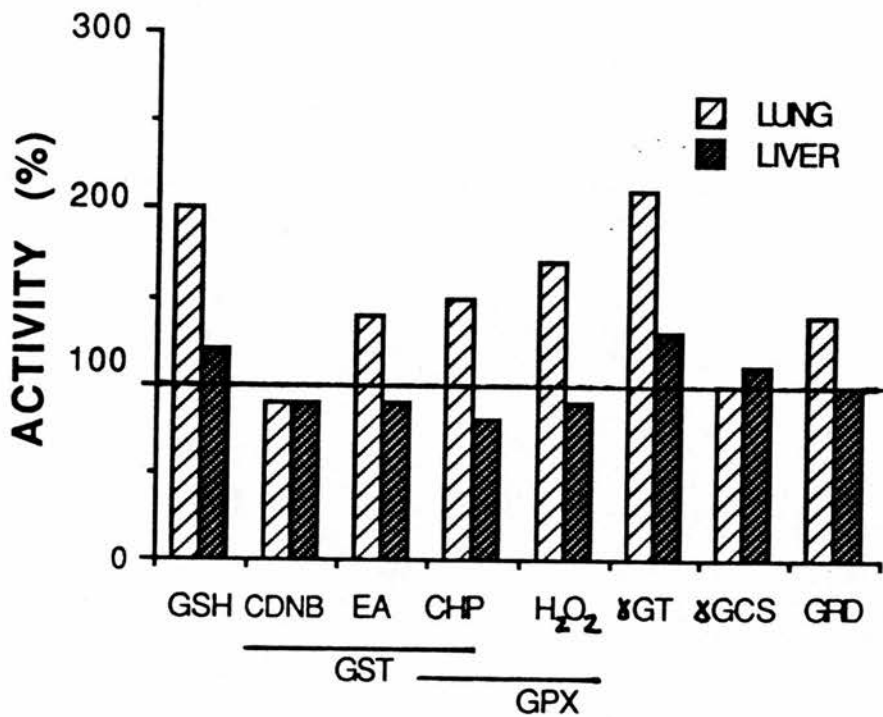
Table 12: GSH and glutathione-dependent enzyme expression in rat lung and liver tissues, hyperoxic and control.

	GSH	Activity							
		GST		GPX		γ GT	γ GCS	GRD	
		CDNB	EA	CHP	H ₂ O ₂				
Lung	Control	1.21±0.089	+0.17±0.006	0.01±0.005	0.11±0.015	0.09±0.013	1.38±0.412	0.03±0.095	0.09±0.032
	Hyperoxic	*2.32±0.328	0.12±0.025	0.007±0.001	+0.17±0.29	*0.14±0.008	**2.95±0.352	0.03±0.003	0.06±0.018
Liver	Control	4.60±0.723	1.38±0.322	0.03±0.006	0.49±0.060	0.36±0.075	1.17±0.727	0.05±0.004	0.11±0.012
	Hyperoxic	5.32±0.276	1.23±0.124	0.03±0.007	0.41±0.121	0.33±0.106	1.54±0.696	0.05±0.013	0.11±0.037

GSH concentration is expressed as μ mol/g tissue. Enzyme activities are expressed as μ moles/min/mg protein except γ GT which is expressed as μ mol/min/g tissue. Other details are given in Materials and Methods (Section 2.0). The abbreviations used are as Table 7. The values shown are means \pm standard deviations, n= 3 for lung samples (pooled) and n = 5 for liver samples. * Significantly different from control p<0.005. ** Significantly different from control p<0.01, +Significantly different from control p<0.05.

FIGURE 28 Relative Expression of Glutathione-Dependent Enzymes in Hyperoxic Exposed Rat Lung and Liver Tissues Following Exposure to High Oxygen.

The values shown are expressed as percentages of the control. See Table 11 for further details.



substrate. GST activity towards ethacrynic acid was found to be unchanged in both the samples.

The evidence therefore indicated there was no change in GST composition as a consequence of hyperoxia. The protein staining pattern of both the liver and lung samples clearly showed no major elevations of any GST subunits under hyperoxic conditions (Figure 29). In order to confirm this finding, Western blot analysis was carried out using antibodies raised against the rat GST subunits of the three main GST families; alpha (Yc, Ya, Yk); mu (Yb₁, Yn) and pi (Yf).

Using the specific antibody raised towards the rat Yc subunit, no difference in the expression of this protein was found, either in lung or liver, under high oxygen conditions (Figure 30). The antibody raised towards the rat Ya GST, however, was also found to cross react with the Yc protein present in the lung and liver samples, and also with the purified alpha Yc/Ya standard. This blot using the antibody towards the Ya subunit (Figure 30) indicated that very little Ya GST was found to be present in the lung, but was the major protein present in the liver. In both tissues, however, this subunit was unaffected by hyperbaric conditions. In contrast, the Yk GST protein was found to be suppressed in the high oxygen treated rat lung, but unchanged in the liver. This protein was found at higher levels in the lung than in the liver (Figure 30).

In the control and high oxygen treated rat lung samples, no expression of the Yn protein was found, and no change in the hepatic level of this subunit was observed (Figure 31). A slight decrease in the Yb subunit was detected in the hyperbaric lung samples. In the liver, no change in the expression of this subunit was observed. Interestingly, an induction of the Yf protein was found in high oxygen treated rat livers, but no difference was detected in the lung samples compared with the liver (Figure 32).

FIGURE 29 Protein Staining Pattern for Hyperoxic and Control
Exposed Rat Lung and Liver Tissues.

Individual rat liver and pooled rat lung samples were separated by SDS-PAGE. 50µg of soluble protein was taken per track. Mobilities of Yc, Yb and Yf standards are also shown (STD). Hyperoxic and control treated lung and liver samples are as indicated.

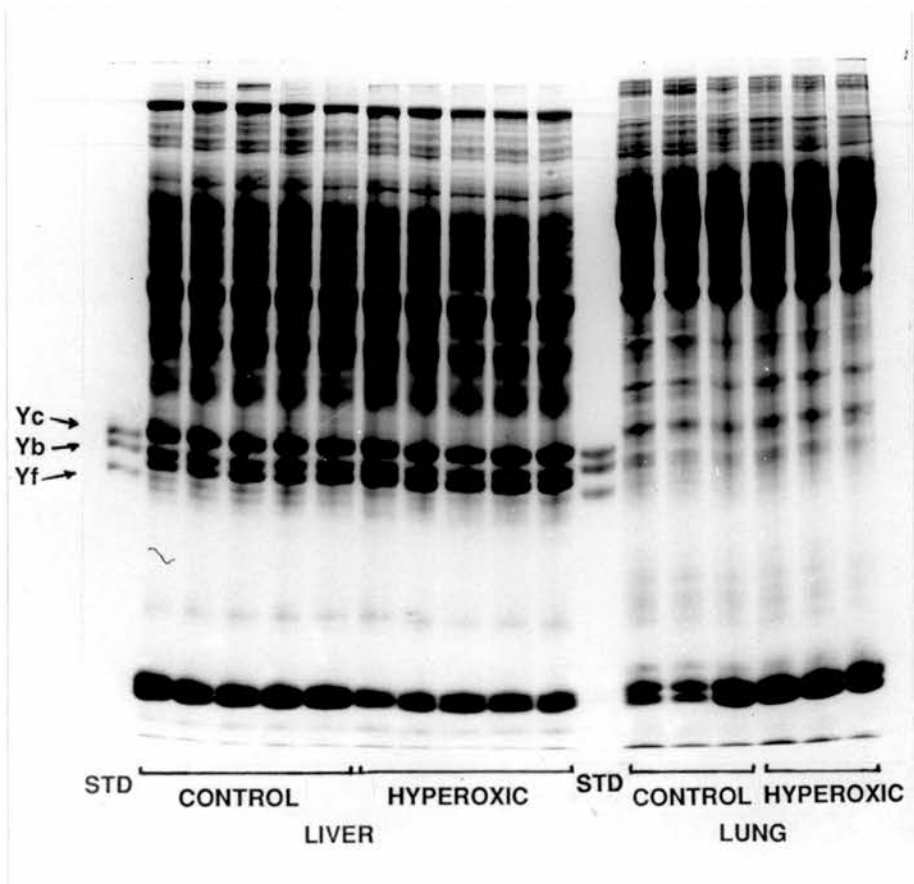
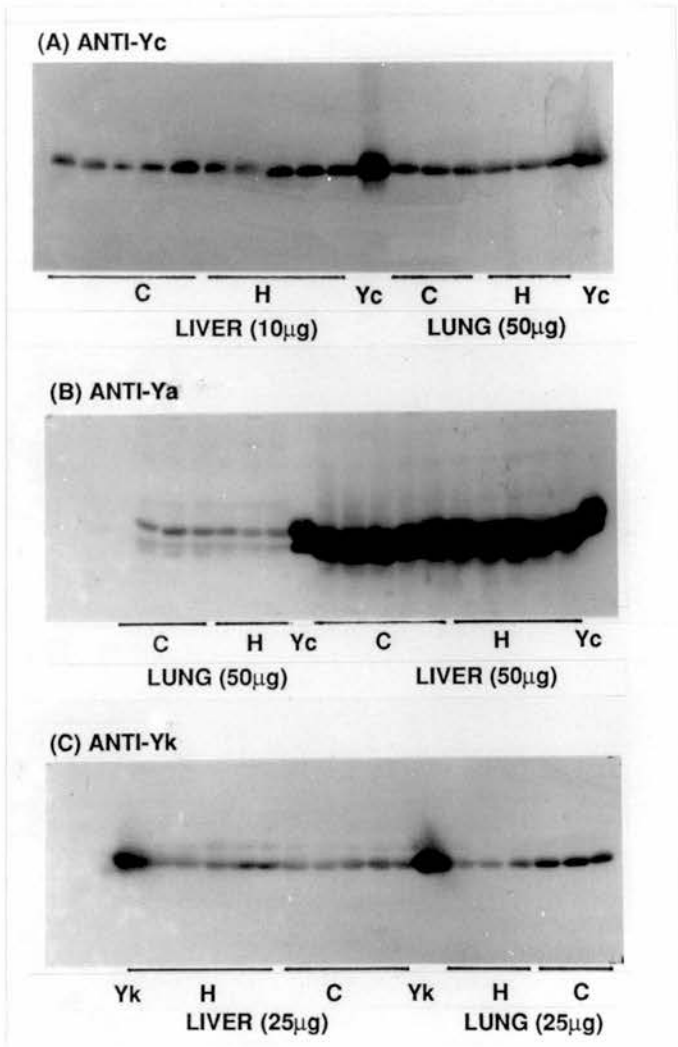


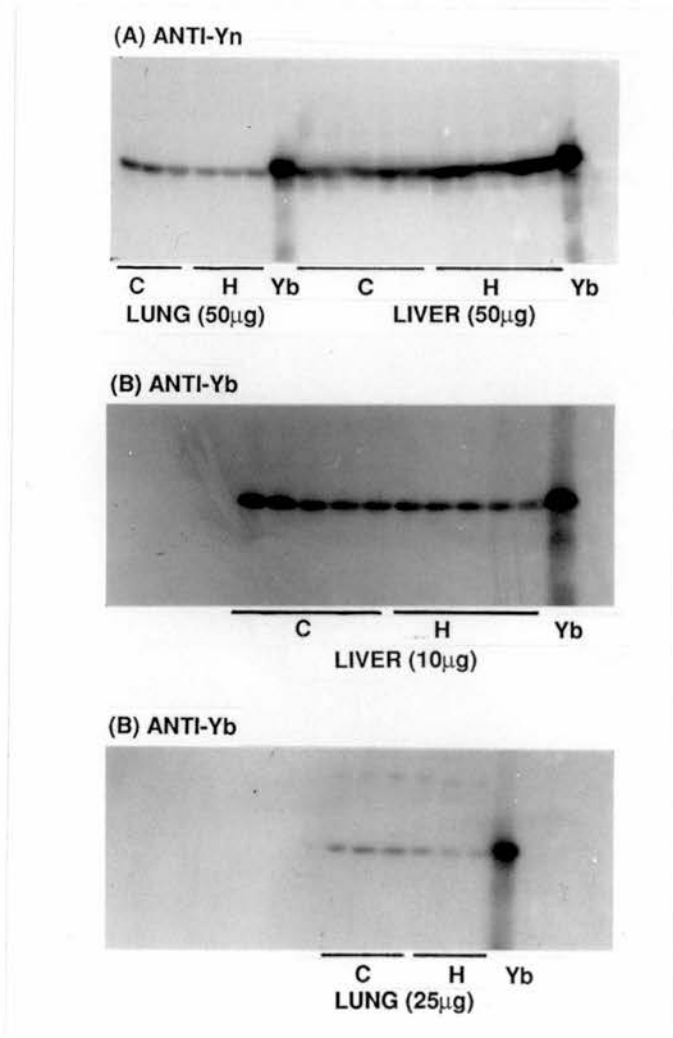
FIGURE 30 Glutathione S-transferase Isoenzyme Expression In
Hyperoxic and Control Exposed Rat Lung and Liver
Tissues using Antibodies Raised against Alpha Class
Proteins.

Amount of soluble protein was loaded as indicated, subjected to SDS-PAGE, transferred to nitrocellulose and probed with specific rat antisera raised against a) Yc, b) Ya and c) Yk subunits. Standards used were indicated by subunit designation. C is control and H is high oxygen treated rat and liver samples. Bands were visualised using ¹²⁵I protein A, and autorads developed at various time periods. Other details are described in Materials and Methods (Section 2.0).



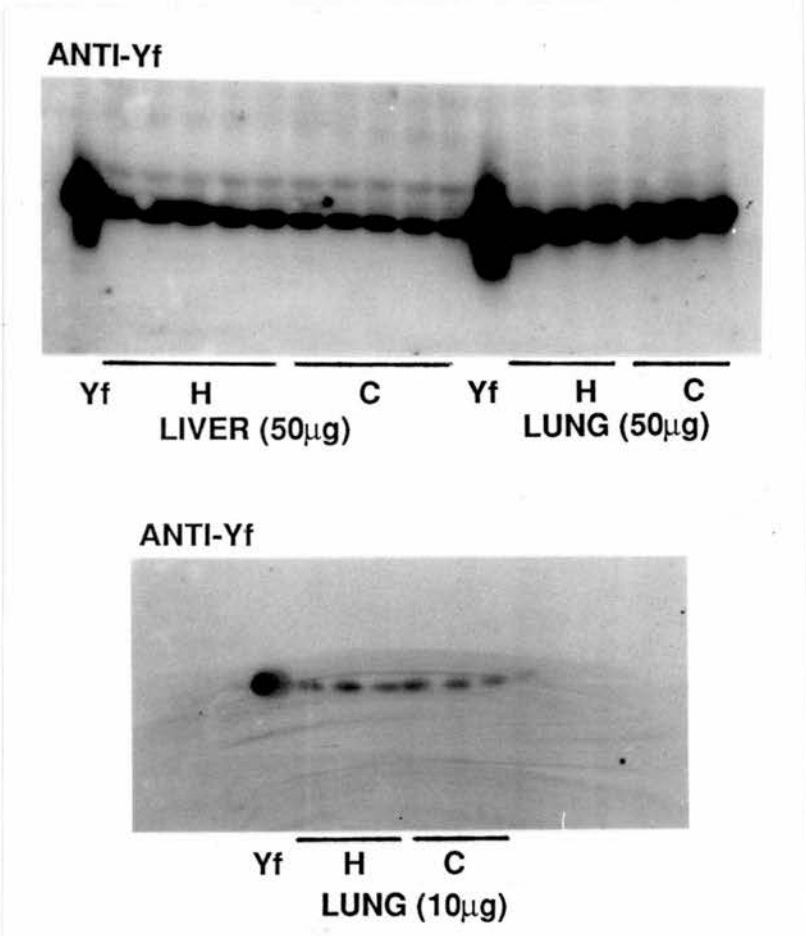
**FIGURE 31 Glutathione S-transferase Isoenzyme Expression in
High oxygen and Control Exposed Rat Lung and Liver
Tissues using Antibodies Raised against Mu Class
Proteins.**

Amount of soluble protein was loaded as indicated, subjected to SDS-PAGE, transferred to nitrocellulose and probed with specific antisera raised against a) Yn, and b) Yb₁ subunits. Standards used were indicated by subunit designation. C is control and H is high oxygen treated rat lung and liver samples. Bands were visualised using ¹²⁵I protein A, and autorads developed at various time periods. Other details are described in Materials and Methods (Section 2. 0).



**FIGURE 32 Glutathione S-transferase Isoenzyme Expression in
High Oxygen and Control Exposed Rat Lung and Liver
Tissue using Antibodies Raised against Pi Class
Proteins.**

Amount of soluble protein was loaded as indicated, subjected to SDS-PAGE, transferred to nitrocellulose and probed with specific antisera raised against rat pi class Yf subunit. Standard used was rat Yf and is indicated as such. C is control and H is high oxygen treated rat lung and liver samples. Bands were visualised using ¹²⁵I protein A, and autorads developed at various time periods. Other details are described in Materials and Methods (Section 2. 0).



Reduced GSH, which is an important cofactor in the protection of cells against toxic oxygen molecules, was significantly elevated in high oxygen treated rat lung tissues 2.0 fold ($p < 0.005$), and was also slightly higher in the hyperbaric liver samples, but not significantly. It was intriguing that, in conjunction with the elevation in GSH, γ GT was also considerably elevated (2.1 fold, $p < 0.01$). The other glutathione-dependent enzymes, γ GCS and GRD, were found to be unchanged in both the lung and liver high oxygen treated rat samples, compared with the controls (Table 12, Figure 28).

4.2.3 Discussion.

Reduced GSH value, determined for the control liver, was similar to those reported in the literature, but at the lower end of the range, 5-7 μ mole/g tissue (Sies et al, 1983). The normal lung GSH level was equivalent to that described by Meister (1983) 1.5 μ mole/g tissue, while γ GT, GRD and GPX activities towards cumene hydroperoxide and hydrogen peroxide, were all similar to those reported by Jenkinson et al (1987).

Enhanced resistance to oxygen induced damage, by pretreatment of lung cells with a lower exposure of oxygen, has been shown by Kimball et al (1976; Sedgwick & Robins, 1980; Christman et al, 1985; Morgan et al, 1986; Shelton et al, 1986). These workers correlated this effect with an increase in reduced GSH superoxide dismutase, and also the enzymes glucose 6-phosphate dehydrogenase, glutathione reductase and GPX. In agreement with this work, a similar elevation in GPX activity was observed here. This increase in GPX activity was confined to the selenium-dependent enzyme, as no change in the GST mediated activity was measured. Reduced GSH was also significantly elevated in the lung tissue as a consequence of hyperbaric oxygen exposure. This is also in agreement with the findings of Kimball et al (1976). Hepatic GSH levels was not altered in treated animals in contrast.

GSH levels form an important part of a protection mechanism against oxygen toxicity, by either reacting directly with oxygen radicals, or acting as a cofactor for glutathione-dependent enzymes which remove peroxides. The increase in GSH levels as a consequence of hyperbaric oxygen therefore appears to be an important part of the adaptive response to elevated oxygen conditions. Concomitant with the increase in reduced GSH, a significant elevation in γ GT was observed. It is of interest, that in almost all cases where GSH is elevated, γ GT is also changed. In contrast to the findings of Kimball *et al* (1976), no increase in GRD activity was found. It is possible that rat strain differences could account for this discrepancy, although unlikely. It is probable, however, that the changes in GRD, if any, are extremely small, as only a marginal induction was observed in the study of Kimball *et al* (1976).

Rat lung GST activity measured here towards CDNB and ethacrynic acid was similar to that reported by Hayes & Mantle (1986b). ^{The GST activity using as a substrate} CDNB was significantly lower in animals exposed to elevated oxygen concentrations. In agreement with a study carried out by Hayes & Mantle (1986b), where the distribution of different GST subunits was determined in liver and various extrahepatic tissues, such as lung, different subunits were found to be expressed at differing levels in the control lung and liver tissues. Yb₁ subunit was found to be expressed at higher levels in the liver than the lung. Another mu class protein (Yn), however, was only present at very low levels in the liver, and not detectable in the lung. The pi class Yf subunit was expressed in high levels in the lung, but only at low levels in the liver. In contrast the alpha Ya subunit was, in agreement with the findings of others (Mannervik *et al*, 1985; Hayes & Mantle, 1986b), the major form present in rat liver, with only a small amount of Ya detected in the lung. The other alpha class GST subunits, Yc and Yk were found in both the lung and liver, with Yk higher in the lung, and Yc probably higher in the liver.

These alpha class GST subunits have been shown to have high non-selenium dependent GPX activity; therefore any changes in the profile of these isoenzymes under high oxygen conditions would be perhaps important in mediating the protection of the cells to high oxygen concentration.

Under hyperbaric oxygen conditions, changes in the levels of GST subunit expression in both rat lung and liver were observed. Surprisingly, however, an induction of the pi class Yf subunit was observed in the liver. The reason for this induction is unclear, but it is possible that either oxygen metabolites direct, or byproducts produced in the lung or liver alter the hepatic level of expression of this protein. It is interesting that it is this protein which is induced in the liver in preneoplasia, and also, that as yet, very few inducers of this subunit have been found. The enzymatic data did not, however, show a corresponding increase in either CDNB or ethacrynic acid activity. In the lung, a significant decrease in Yk GST subunit was detected. This protein, which was purified by Hayes (1986) is characterised by low CDNB and high ethacrynic acid activity. This isoenzyme is thought to play a role in cellular protection against lipid peroxidation, as it has been shown to have high activity against toxic lipid peroxidation products, such as 4-hydroxyalkenals (Danielson *et al*, 1987). It is therefore, of interest, that this particular GST subunit was suppressed in the high oxygen exposed lung samples, while the selenium-dependent GPX activity was significantly elevated, indicating, perhaps speculatively, that some form of relationship between these types of enzymes exist. It may also indicate that the role of Yk in the protection against lipid peroxidation may not be as important as first thought. The apparent intriguing inverse relationship between selenium-dependent GPX and GST in an adaptive response is worthy of further study. It is possible that selenium plays a role in the regulatory mechanism.

Selenium levels are known to affect the levels of expression of these two types of GPX enzyme (Masukawa et al, 1983) and could be a factor. For example, oxidative stress may influence selenium levels and, in turn, influence the distribution of the selenium and non-selenium dependent forms of GPX. It would have been interesting to have investigated the changes observed in rats fed selenium deficient diets exposed to high oxygen conditions. In addition the exposure of cells in culture to H₂O₂, in the presence and absence of selenium, may have given further clues to the possible relationship. Whether stable expression of these factors exist in cell lines made resistant to high oxygen (oxidative stress) would also have been interesting to investigate.

It is important to note that other oxygen induced adaptive enzymes may also be very important in the resistance mechanism against high oxygen stress (Kimball et al, 1976). These enzymes include superoxide dismutase and glucose 6-phosphate dehydrogenase. In addition, however, the study described here has shown that GSH and glutathione-dependent enzymes, such as γ GT and GRD, are induced as an adaptive response to oxygen, and may form part of a more general response. This finding is strengthened by the knowledge that it has been previously shown that GSH and glutathione-dependent enzyme, such as selenium-dependent GPX, are induced in bacteria in response to oxidative stress. In particular this adaptive response in bacteria to H₂O₂ involves the induction of a series of proteins including catalase and GRD, under the regulation of oxyRI regulon (Christman et al, 1985; Morgan et al, 1986) (see also Section 5.1.0).

4.3.0 Resistance of Liver Cells to Carcinogens

4.3.1 Introduction.

Chemical hepatocarcinogenesis is known to be a multistep process in vivo (Pitot & Sirica, 1980; Farber, 1984b). The sequence of events is believed to be initiation, followed by promotion of some initiated cells. Major

conceptual problems exist, however, when ^{the} mutation-selection concept is used to explain this multistep nature of the carcinogenic process, and the mechanistic role or position of precancerous lesions. If the mutational events occurring during the process of initiation have generated an initiated cell, which displayed 'spontaneous' growth, then the multistep carcinogenic process could be explained by the progression of new genetically altered cells for increasing ability to grow 'spontaneously'. Unfortunately, this hypothesis is not consistent with the many observations in this area in the literature (Farber, 1984b). For example, efficient initiation of carcinogenesis with a relative small dose of carcinogen does not generate any altered cells with any growth autonomy, thus reflecting the well established need for another operation such as a promoting environment.

An alternative hypothesis to the random mutation-selection theory, is the physiological adaptation concept (Farber, 1984a; 1984c). In this theory, carcinogenesis is divided into stages, and the long pre-cancerous phase of cancer development is described as a form of adaptation of the cells to a hazardous environment. In particular, this form of acute adaptation to xenobiotics, such as carcinogens, is thought to occur through the induction of new enzyme patterns which are related to their metabolism and detoxification (Farber, 1984a; 1984c). This adaptation concept has mainly been studied in rat liver carcinogenesis models.

There are many different models of liver carcinogenesis but the most commonly studied are those induced by chemical mutagens. The Solt-Farber model (Solt & Farber, 1976) describes the commonly used strategies for inducing liver carcinogenesis. The preneoplastic foci induced by chemical carcinogens, have been shown to have adapted to the cytotoxic environment by altering their enzymatic profile. Biochemically, the preneoplastic foci have elevated levels of various phase II drug metabolising enzymes (e.g. GST, DT-

diaphorase and epoxide hydrolase); however, phase I metabolising enzymes (cytochrome P450's), which are often involved in activation of carcinogens, are depressed (Cameron et al, 1976; Roomi et al, 1985). Enhanced GSH content is also found in the high γ GT positive, preneoplastic foci (Demi & Oesterle, 1980). This elevation in GSH, γ GT and phase II drug metabolising enzymes is consistent with the observation that persistent hepatocyte foci are more resistant to the cytotoxic effects of carcinogens, as compared to normal liver (Spiewak & Eriksson, 1983; Spiewak, 1984; Roberts et al, 1983).

GST expression in preneoplastic foci and in neoplastic nodules was studied here to establish similarities between this method of adaptive response, relative to the others described in this thesis. The glutathione-dependent enzymes γ GCS, selenium-dependent GPX and GRD were also studied in these models.

4.3.2 Results.

After the induction of γ GT positive preneoplastic and neoplastic nodules using aflatoxin B₁ (AFB₁), as determined by Dr. M. Manson, M.R.C. Toxicology Unit, Carshalton, using histochemical staining, GST activities towards the general substrate CDNB were markedly increased 1.9 and 2.5 fold respectively, compared to control rat liver (Table 13, Figure 33). Other model substrates, such as ethacrynic acid and cumene hydroperoxide were also used in this study.

Although other classes of GST isoenzymes can utilise the substrate ethacrynic acid (EA), this substrate tends to be diagnostic for pi class Yf isoenzyme activity. In preneoplastic foci, pi class activity using this substrate was elevated 2.3 fold, but an even higher 9.4 fold increase was observed in the rat neoplastic nodules. The peroxidase activity towards cumene hydroperoxide in both preneoplastic foci and neoplastic nodules was decreased. This decrease in peroxidase activity appeared to be due to a decrease in selenium-dependent

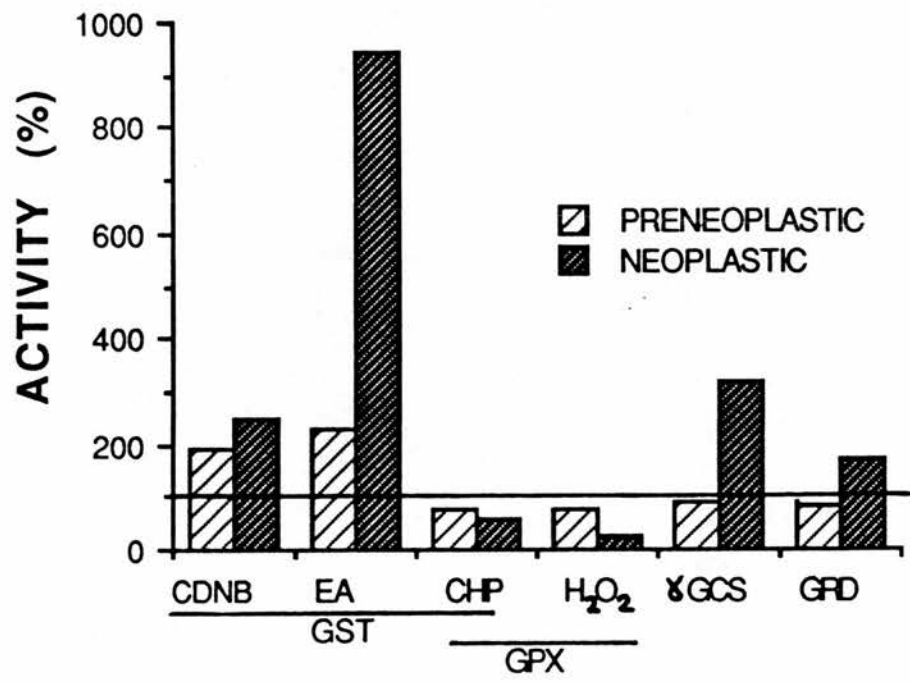
Table 13: Glutathione-dependent enzyme expression in control, preneoplastic and neoplastic foci from rat liver.

	Activity					
	GST		GPX		γ GCS	GRD
	CDNB	EA	CHP	H ₂ O ₂		
Control	5.0	0.025	0.295	0.221	0.041	0.168
Preneoplastic	9.5	0.058	0.229	0.169	0.036	0.141
Tumour A	14.2	0.238	0.178	0.044	0.133	0.242
Neoplastic Tumour B	11.7	0.237	0.156	0.064	0.127	0.325
Average	<u>12.5</u>	<u>0.238</u>	<u>0.167</u>	<u>0.054</u>	<u>0.130</u>	<u>0.283</u>

The abbreviations used are GST, glutathione S-transferase; GPX, glutathione peroxidase; γGCS, γ glutamyl cysteinyl synthetase; GRD, glutathione reductase; CDBN, 1-chloro-2,4,dinitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide. All reactions were carried out at 37°C as described in Materials and Methods (Section 2. 0). Values are expressed in μmoles/min/mg protein. Tumour A and B represent different tumours taken from one rat, the average for the two values is given in parenthesis. Control and preneoplastic values are derived from one rat liver.

FIGURE 33 Relative Expression of Glutathione-Dependent Enzymes
In Rat Preneoplastic Foci and Neoplastic Nodule Cells.

The values shown are expressed as percentages of the control. Value taken for the neoplastic nodule cells is the average of both tumour A and B determinations as indicated in Table 12. See Table 12 for further details.



GPX activity, as the activity of this enzyme to the substrate hydrogen peroxide was also decreased. A more marked decrease in this activity was observed, however, in the neoplastic nodules compared to the preneoplastic foci.

As non-selenium dependent GPX enzymes cannot metabolise hydrogen peroxide as a substrate, an overall 1.5 fold increase in non-selenium dependent GPX activity was calculated to occur in neoplastic nodules. This activity tends to be associated with alpha class GST isoenzymes.

The evidence, therefore, seemed to show that significant elevations, in particular GST isoenzymes, seemed likely in both preneoplastic foci and neoplastic nodules. The comparison of the protein staining patterns of control liver, preneoplastic foci and two neoplastic nodules, Tumour A and B taken from one rat, indicated dramatic changes in the GST isoenzyme profile in the preneoplastic foci and neoplastic nodules compared to the control liver (Figure 34). In particular, a large increase in a protein with a mobility equivalent to Yf subunit was observed in both the neoplastic nodules A and B. Other changes in GST isoenzyme levels, especially in alpha Ya subunit could also be seen. In order to confirm these changes in GST isoenzyme profiles, Western blot analysis was carried out using antibodies raised against rat GST subunits from the three main GST classes; alpha Yc and Ya, mu Yb and pi Yf.

The results from the Western blots confirmed, as indicated in Figure 35, that there were changes in GST isoenzyme profile between control liver samples, and those from preneoplastic foci and neoplastic nodules. Using antibodies raised against the Yc subunit, no difference in expression of this subunit was observed in the preneoplastic foci, or neoplastic nodules. In contrast, however, Ya subunit levels were increased in the neoplastic nodules compared to the control liver and preneoplastic foci samples. Similarly, an increase in Yb GST was also detected in the neoplastic nodules. In particular, however, an elevation in the Yf GST subunit, which is normally only detected at

FIGURE 34 Protein Staining Pattern for Preneoplastic Foci,
Nepolastic Nodule and Control Rat Liver.

Samples were separated by SDS-PAGE. 50µg of soluble protein was taken per track. Mobilities of rat Yc, Yb₁ and Yf are indicated. C represents control; P, preneoplastic and TA and TB neoplastic nodules A and B respectively.

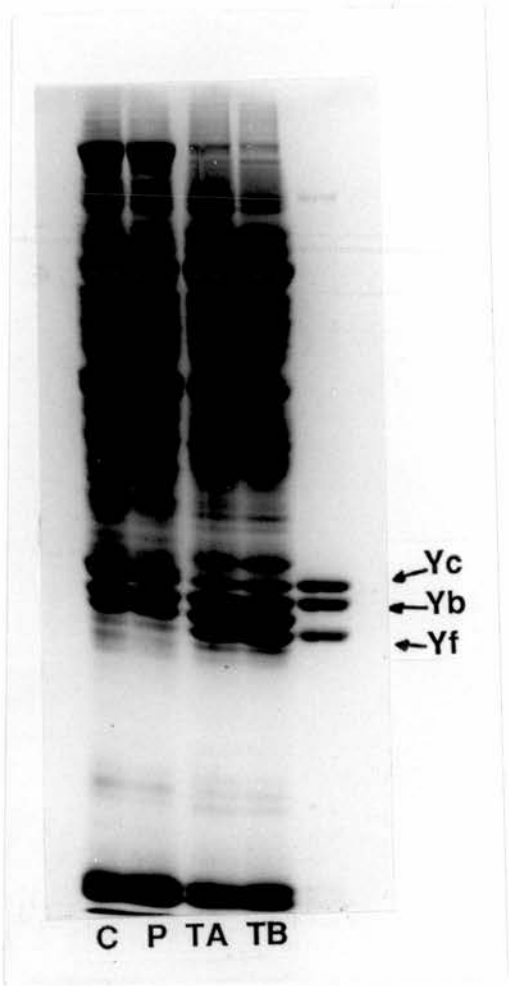
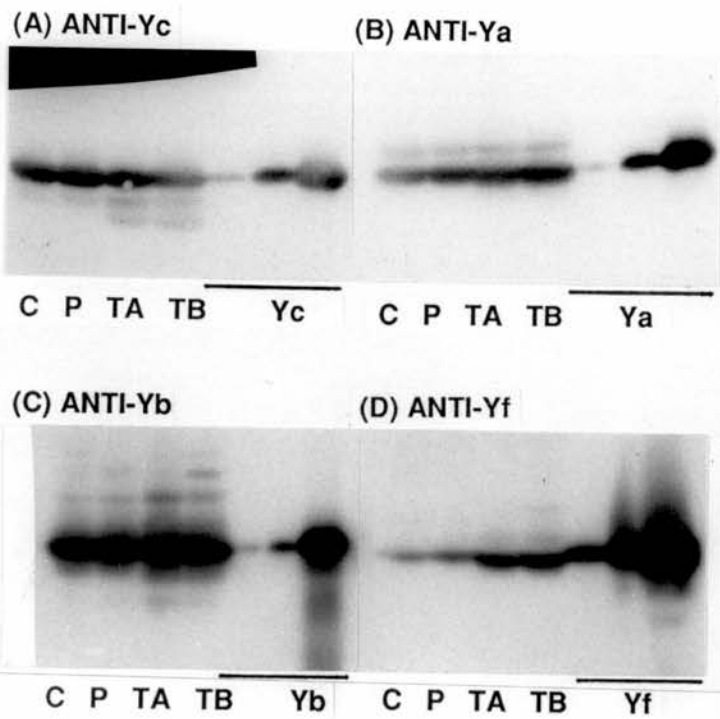


FIGURE 35 Glutathione S-transferase Isoenzyme Expression in
Preneoplastic Foci, Neoplastic Nodules and Control Rat
Liver.

50µg of soluble protein was loaded, subjected to SDS-PAGE transferred to nitrocellulose and probed with specific antisera raised against rat:

- a) Yc; b) Ya; c) Yb₁ and d) Yf subunits.

Standards used were as indicated by subunit designation. C is control, P is preneoplastic foci, and TA and TB are neoplastic nodules A and B respectively. Bands were visualised using ¹²⁵I protein A, and autorads developed after 8 hours. Other details are described in Materials and Methods (Section 2.0).



low levels in control rat liver, was seen in both preneoplastic foci and more markedly, in the neoplastic nodules. This change in GST isoenzyme profile may therefore influence the response of the preneoplastic foci and neoplastic cells to the effects of carcinogens and cytotoxic compounds. Other glutathione-dependent enzymes may, however, also influence the response of these cells to carcinogens, and as a consequence these were studied in control, preneoplastic foci and neoplastic cells also.

γ GCS and GRD are both glutathione-dependent enzymes which are involved in maintaining reduced GSH levels within cells. Only in neoplastic nodule cells were γ GCS (3.2 fold) and GRD (1.7 fold) elevated compared to control activities. No changes in these enzymes were observed for preneoplastic foci derived cells (Table 13, Figure 33).

4.3.3 Discussion.

It was documented in all the different models of rat liver carcinogenesis that a well defined sequence of a variety of tissue and cellular changes can be observed. These include the focal proliferation of hepatocytes, hepatocyte nodules, hyperplastic nodules, neoplastic nodules, adenomas and, finally, the formation of a malignant tumour. In the study described here, glutathione-dependent enzyme expression was determined in an AFB₁ induced neoplasia and, in particular, in two different progressive stages of carcinogenesis i.e. in preneoplastic foci and neoplastic nodules. In this regard, Kitahara et al (1984) and Sato et al (1984; 1987) have carried out the most detailed studies into the changes in GST isoenzyme profiles during hepatocarcinogenesis.

It was not clear, however, from ^{the studies of} Kitahara et al (1984), which stages of hepatocarcinogenesis were taken, except that livers with foci and nodules were investigated. In this study, Kitahara et al (1984) indicated by purification, SDS-PAGE and two dimensional SDS-PAGE that, during early hepatocarcinogenesis, an elevation in Yb, Ya and Yf subunits were observed,

with no change in Yc subunit. Pi class Yf was also shown to be elevated in preneoplastic lesions by Western blot analysis, and proposed to be a useful preneoplastic marker enzyme for chemical hepatocarcinogenesis by Sato et al (1984; 1987). Overall, Kitahara et al (1984) found an increase in GST activity, towards the substrate CDNB, of 2 fold in the hepatocarcinogenic livers. In agreement with this finding, I found a 1.9 fold and a 2.5 fold elevation in GST activity in preneoplastic foci and neoplastic nodules respectively. This change in GST activity was associated with an increase in both Yf subunit levels, and activity towards ethacrynic acid as a substrate in preneoplastic foci cells. The change in GST profile pattern was much more dramatic for the neoplastic nodule cells, with an elevation in the Yf GST subunit observed not only by Western blot analysis, but also by SDS-PAGE and enzymatic assay. An increase in Yf and Ya GST subunits, and activity for the alpha class GST, was found in the neoplastic nodule liver also; this was in complete agreement with the elevations observed by Kitahara et al (1984) using their two dimensional gel electrophoresis system. Pickett et al (1987) has also shown, at the molecular level, an increase in message transcript for Ya (2.8 fold) and Yb (5.3 fold) by Northern blot analysis, which again fits in with findings observed here in this study at the protein level. As GST's have been shown to be involved in the detoxification of carcinogens (Smith et al , 1977; see also Section 1.10.6), elevation in these isoenzymes within the preneoplastic foci and neoplastic nodules would probably play an important role in the resistance of these cells to the effects of the carcinogens. Other glutathione-dependent enzymes are also known to be involved in drug detoxification (Larsson et al, 1983a; Wolf et al, 1987a, 1987c; Hayes & Wolf, 1988) and therefore may also be involved in the resistance mechanism.

The expression of other glutathione-dependent enzymes such as GPX, γ GCS and GRD, which are involved in the maintenance of reduced GSH levels,

have not been studied to my knowledge in early hepatocarcinogenesis. In the study described here, however, an increase in γ GCS and GRD activities were found in neoplastic nodules. Interestingly, a decrease in selenium-dependent GPX activity in both preneoplastic foci, and particularly in neoplastic nodules, was seen. As the alpha Ya GST (exhibits non-selenium dependent GPX activity) was shown to be elevated in the neoplastic nodules, this again raises the possibility of an inverse relationship between non-selenium dependent and selenium-dependent GPX activity in cells. Whether this is due to a direct effect of the carcinogen itself, or an effect of the carcinogen on selenium levels within the nodules, which in turn influences the distribution of the two different forms of GPX is at present still unclear.

The preneoplastic foci and neoplastic nodules were induced by AFB₁: it would have been interesting to have extended this to include other induction model systems. Comparison of data regarding GST isoenzyme expression, however, with that of Kitahara et al, (1984), who used diethylnitrosamine and 2-acetyl aminofluorene to induce hepatocarcinogenesis, indicates that the changes observed are a general response. The most studied changes in the different experimental models of induction of chemical hepatocarcinogenesis are in γ GT and reduced GSH. In a study by Roomi et al (1985), in all four of the models he looked at, there was a consistent elevation of γ GT and GSH. In particular, Deml & Oesterle (1980) have demonstrated that enhanced GSH content occurs in the histochemical γ GT positive enzyme altered islands induced by carcinogens. This finding further exemplifies the theory that γ GT is important in maintaining reduced GSH levels, especially in drug resistant cells (Ahmad et al, 1987). γ GT positive histochemical staining was identified in both the preneoplastic foci and neoplastic nodules used here by Dr. M. Manson, M.R.C. Toxicology Unit, Carshalton, but, unfortunately, as the samples were prepared in the MRC Unit, it was not possible to determine GSH levels in these

samples. It would have been of interest to look at other models of liver carcinogenesis not involving chemical mutagens, such as the choline-methionine-deficient model (Rogers & Newberne, 1975; Sells et al, 1979).

In summary, the adaptive response in liver cells to a carcinogen involves the induction of a series of glutathione-dependent enzymes, including the GST's which are known to be involved in drug detoxification. In particular, a dramatic elevation in the Yf GST isoenzyme was observed. This isoenzyme was demonstrated to be elevated in cell lines made resistant to anticancer drugs (Batist et al, 1986), and also to be highly expressed in tumours which tend to show resistance to chemotherapy (Carmichael et al, 1988). Other GST's, such as Yb₁ and Ya, were elevated in this model also. Interestingly, selenium-dependent GPX activity was decreased in the neoplastic nodules studied. The changes in glutathione-dependent enzyme expression (and also other drug detoxification enzymes) in the preneoplastic foci, and particularly in neoplastic nodules, would probably be important in making these liver cells resistant to the effects of carcinogens, giving them a selective advantage for growth. A second initiation or factor would, however, be involved in the final progression of the cells to a tumour.

The aim of this study was to investigate whether there was a common underlying mechanism of drug resistance involving GSH and glutathione-dependent enzymes, especially in the acquired drug resistance of tumour cells to cytotoxic drugs, and also whether this mechanism could be related to an adaptive response as a consequence of the administration of a cytotoxic compound, or to a selection for intrinsically resistance cells. The evidence presented in this thesis shows that GSH and glutathione-dependent enzymes were altered in an adaptive response to chemical induced insult (Table 14) in all the drug resistant models investigated, and, therefore, may form part of a general protection mechanism against a cytotoxic environment. This has enabled the generation of a model which can be compared with other known adaptive responses, such as heat shock, viral infection, alkylating agent induced DNA repair and oxidative stress. By looking at how these other adaptive responses are regulated, it will be possible to generate ideas about how the adaptive response described here for GSH and glutathione-dependent enzymes, is regulated.

5.1.0 The Adaptive Response

The best characterised adaptive response at a molecular level is that of heat shock (for reviews see Burdon, 1986; Lindquist, 1986). Cultured cells, or, indeed, whole organisms, respond to elevated temperatures by synthesising a number of highly conserved proteins, the heat shock proteins or hsp's. This response is universal, and has been observed in every organism in which this response has been looked for so far. The proteins can also be induced by a variety of other agents, including ethanol (Li et al 1983) and heavy metals (Burdon, et al 1982), and seem to play a role in normal growth and development (Burdon, 1986). The regulation of hsp's genes has been studied by the deletion of various DNA sequence to determine the position and nature of the DNA

TABLE 14 Summary of glutathione-dependent enzyme expression in four drug resistant models.

MODEL	GST					GPX		GSH	γGT	GRD	γGCS
	CDNB	EA	Non Se		CHP	H ₂ O ₂					
			GPX								
Acquired drug Resistance) CHO	↑↑	↑	↑↑	↑↑	NC	↑↑	↑↑	NC	NC	
) Ovarian	↑↑	NC	↑	↑↑	↑↑	↑↑	↑↑	↑	NC	
) Sarcoma	↑↑	NC	↓	NC	↑↑	↑	↑↑	NC	NC	
Normal Cells Resistant to chemotherapeutic drugs) Bone marrow	↑↑	NC	↑↑	↑↑	NC	↑↑ ^a	NC	NC	NC	
) Granulocytes	↑↑	NC	↑	↑	NC	↑↑ ^a	NC	NC	NC	
) Lymphocytes	NC	NC	NC	NC	↑	NC ^a	↑↑	↑↑	NC	
Resistance of normal cells to oxygen) Lung	↓↓	NC	NC	↑	↑↑	↑↑	↑↑	NC	NC	
)										
)										
Resistance of liver cells to carcinogens) Preneoplastic	↑↑	↑↑	↑	NC	↓	↑↑ ^b	↑↑ ^c	NC	NC	
) Neoplastic	↑↑	↑↑	↑↑	↓	↓↓	↑↑ ^b	↑↑ ^c	↑↑	↑↑	
)										

↑↑ indicates a rise or fall in activity respectively; ↑, ↓ indicates a slight change; NC, represents no change; ^a as determined by Adams *et al.* (1985); ^b determined by Roomi *et al.* (1985); ^c determined in collaboration with M. Manson, MRC Toxicology Unit, Carshalton. Determinations are made with reference to the appropriate controls in each model. Abbreviations used are as described for Table 13. Non-selenium dependent GPX activity (Non se. GPX) which is associated with mainly alpha class GSTs is calculated from the difference between total GPX activity towards CHP, and selenium dependent GPX activity towards H₂O₂ (Carmagnol *et al.* 1983).

sequence elements responsible for regulating the induction of hsp gene transcription (Pelham, 1985; Ayme et al, 1985). In drosophila, the organism in which heat shock has been most studied, a conserved sequence located 20 nucleotides upstream of the promoter region has been identified (Pelham, 1982). This sequence shows homology with upstream sequences of other drosophila hsp genes, and a minimal consensus sequence has been derived: C--GAA--TTC--G. This is referred to by Pelham (1985) as the heat shock element, or HSE. Similar sequences have been found in other species (Pelham 1985). A HSE binding protein, heat shock transcription factor (HSTF), (Parker & Topol, 1984), is, however, essential for the transcription of these genes. This use of a consensus sequence for the regulation of the heat shock adaptive response is similar to the mechanism identified for the effect of interferons (IFNS).

In the case of viral infection of cells, the cells respond by producing IFN's which, in turn, induce a set of proteins which may be involved in the resistance to viral infection, by inhibiting viral replication. In humans the IFN activated genes for these proteins have flanking sequences which, at the 5' end, have a putative consensus signal (Revel & Chebath, 1986), as shown below.

5' TTCN(GC)NACCTC

NGCAGTTTTCTC(CT) TCT-CT 3'

The transcription factor involved in the interaction with these sequences has, as yet, not been isolated. In contrast, however, in the alkylating agent induced DNA repair system in bacteria, the messenger of the induction of the genes has been identified.

The adaptive response in E. coli to low, non lethal dose of an alkylating agent, provides resistance to a higher lethal dose. This has been shown to be under the control of the regulatory ada gene (Jeggo, 1979), and increases the

the ability of the
bacteria to repair alkylating agent induced DNA damage at O⁶ guanine. A second alkylation event to the protein, O⁶ alkyl guanine DNA alkyl transferase, is thought to convert ^{this} ~~the~~ protein to a transcriptional activator for the genes involved in the inducible response to alkylating agents (Teo, et al, 1986; Nakabeppu & Sekiguchi, 1986). This protein has been shown to bind to a specific sequence (AAANNAAAGCGCA) present in the promoter regions of the genes involved in the inducible adaptive response, at a site immediately 'upstream' of the binding site for RNA polymerase (Teo et al, 1986). This inducible adaptive response system to alkylating agents has only been described in bacteria and, in spite of extensive study, there is at present no evidence that it exists in eukaryotic cells.

In the adaptation of bacteria to oxidative stress, the induction of proteins such as superoxide dismutase, catalase, glutathione reductase, and alkylhydroperoxide reductase has been shown. This response is regulated by the positive control of a regulon (OXYR) (Christman et al, 1985; Morgan et al, 1986). This system has also only been found in bacterial systems.

All the evidence suggests that in adaptive response mechanisms, transcriptional activation results from the binding of a transacting factor to consensus sequences 'upstream' of the genes involved. A model for the regulation of the adaptive response to cytotoxic agents involving GSH and glutathione-dependent enzymes, as described in this thesis, may be based on a similar mechanism. Sequences in the 5' flanking regions of the genes for the glutathione-dependent enzymes may provide evidence that this is the case i.e. whether any consensus sequences exist; and also whether DNA binding proteins are induced in response to chemical stress which bind to the 5' flanking regions.

The finding that not all glutathione-dependent enzymes were induced in all the models studied, and also that in some models the changes were

transient, indicates that the regulation mechanism may be complex. Mechanisms other than transcriptional activation, such as mRNA stabilisation, and the influence of external modulators, may also play a role. Many glutathione enzymes are also regulated by complex feedback control mechanisms, and these may also play a role. Interestingly, there appeared to be a relationship between γ GT and GSH levels and selenium and non-selenium dependent GPX activity (see Sections 5.2.0 and 5.3.0).

Adaptive response mechanisms may overlap, indicating common regulatory pathways for certain of the induced proteins. For instance, in heat shocked Chinese hamster HA1 cells, resistance to adriamycin is also observed, (Li et al, 1982) and in oxidative stress in bacteria, certain heat shock proteins are also induced (Christman et al, 1985; Morgan et al, 1986). It would have been important, therefore, if time had permitted, to look at GSH and glutathione-dependent enzymes in heat shocked cells and whether this conferred resistance to chemicals. Another example of overlap in these systems is the finding that the GST can be modulated by IFN's (Adams et al, 1987). Preliminary work, which I carried out with Dr. S. Thomas, showed an elevation in pi class GST in a lymphoblastoma cell line (DAUDI). Further characterisation should be carried out to determine whether GSH and other glutathione-dependent enzymes are also changed.

On the basis of the above discussion, it is possible to suggest that the adaptive response of GSH and glutathione-dependent enzymes in mammalian drug resistant models described here may be a more general response, with similar responses to be found in drug resistant bacteria, plants, insects and yeast.

5.2.0 **Relationship between γ glutamyltranspeptidase (γ GT) and reduced glutathione (GSH) levels.**

One of the interesting findings here was the consistent elevation in γ GT and GSH in almost all the models studied. There are also indications from reports which indicate a relationship between γ GT activity and GSH levels. This has been shown in preneoplasia, in interorgan regulation of GSH levels by γ GT in the kidney, and in drug resistance.

γ GT has been associated with early neoplastic changes in murine and other animal models (Jaken & Mason, 1978; Dawson et al, 1979; Jalenko & Ruoslahti, 1979; Ohmori et al, 1981). Increased expression of γ GT seemed to be a common feature of both preneoplastic and neoplastic lesions of many tissues, including skin, pancreas, (De Young et al, 1978) and mammary carcinomas (Jaken & Mason, 1978; Dawson et al, 1979). In preneoplastic foci in the liver, induced by a variety of different chemicals, large increases in γ GT activity have been reported (Roomi et al, 1985). This data was in agreement with histochemical work reported by many research groups including Solt & Farber (1976) and Cameron et al, (1976). Interestingly, concomitant with elevated γ GT levels, increased GSH levels have also been demonstrated (Deml & Oesterle, 1980). Whether this increase in GSH levels is dependent on the elevation in γ GT activity, and not just due to altered general phenotype of the preneoplastic foci, is at present unclear, although the evidence concerning the involvement of γ GT in the regulation of GSH levels and increase in GSH levels in drug resistant cells, tends to support the former possibility.

The kidney is the tissue which contains the highest level of γ GT (Tate, 1980). It has been demonstrated by Rankin et al (1983) that the kidney is involved in the regulation of plasma GSH levels, with the catabolism of GSH by renal γ GT to form the constituent amino acids for intracellular GSH biosynthesis. The kidney has been shown to have both relatively high levels of

of GSH and a higher GSH turnover rate (Sekura & Meister, 1974) compared to the liver, perhaps as a consequence of the high γ GT levels. The most compelling evidence for a relationship between γ GT levels and GSH levels comes from drug resistant cell lines.

Ahmad et al (1987) demonstrated a correlation between increased levels of GSH within a phenylalanine ^{mustard} drug resistant L1210 cell line, and the increased activity of γ GT. In particular, an increase in the K_m of the γ GT in the drug resistant cell line for extracellular GSH, and an increase in the intracellular cysteine pool was, as a result, (a rate determining factor in GSH biosynthesis) related to the increase in intracellular GSH levels. These experiments would have been more definitive if γ GT inhibitors, such as L-(α S,5S)- γ -amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (AT-125), had been shown to alter GSH levels. The most conclusive experiment on the role of γ GT in modulating GSH levels would have been the expression of a cDNA for γ GT in a γ GT deficient (or low γ GT activity) cell line.

The consistent elevation in GSH levels concomitant with the increase in γ GT activity, together with the finding that tumours tend to have high γ GT activity, allows the possibility to speculate ways of modulating this activity to affect tumour response to chemotherapy. These proposals are discussed in Section 5.4.0.

5.3.0 Relationship between selenium and non-selenium dependent glutathione-peroxidase activity.

The summary in Table 14 indicates an inverse relationship between selenium and non-selenium dependent glutathione peroxidase (GPX) activity. For instance, if selenium dependent GPX activity was increased, non-selenium dependent GPX activity was either unchanged or decreased in the drug resistant model studied and vice versa. The mechanism of this effect, is at present, unknown. It has been demonstrated, however, that selenium concentrations can

influence both GPX enzymes. An increase in selenium level causes an increase in the selenium-dependent form and a decrease in the non-selenium dependent enzyme (GST form) (Kasukawa et al, 1983). Whether selenium levels are modulated to a differing degree by different cytotoxic stresses, such as anticancer drugs and oxygen radicals, to change the levels of the two GPX proteins, has not been studied; this could be a possible mechanism of regulation. The effect of high and low selenium concentrations on the levels of these two forms of GPX in different drug resistant models would be interesting to investigate. Another possible explanation for the inverse relationship between the GPX activities is the differential induction of one of the enzymes in response to a particular cytotoxic stress, and the changes observed, depending on the model studied. This could be through 'activator of repressor elements' at the 5' flanking sequences in the respective genes.

The changes in levels of the two forms of GPX in drug resistance may be important in the overall response to cytotoxic drugs, and may also be important in human tumours. Ways of exploiting these changes and circumvent drug resistance in tumours are discussed below in Section 5.4.0.

5.4.0 Sensitisation of tumour cells to cytotoxic drugs by modulating GSH and glutathione-dependent enzyme levels.

The changes in GSH and glutathione-dependent enzymes may be of central importance in the resistance of tumours to chemotherapeutic agents (Hirono, 1961; Calcutt & Connors, 1963; Suzukake et al, 1982; Wolf et al, 1987a, 1987b, 1987c). On this basis, it is possible to postulate various ways of modulating chemotherapy in order to circumvent drug resistance.

Buthionine sulfoximine (BSO) will deplete GSH levels in tumour cells and sensitise them to anticancer drugs (Green et al, 1984; Somfai-Relle et al, 1984; Hamilton et al, 1985; Crook et al, 1986a; 1986b). The benefits of this

treatment in the clinic may be limited, however, by the serious side effects attributed to this compound. Calvin et al. (1986) indicated that administration of BSO to mice, for example, had led to cataracts. As GSH is involved in normal cellular function, other serious toxic side effects may also be found. In this regard, patients who suffer from GSH deficiency syndromes can suffer from severe nervous system damage, including serious mental retardation (Larsson et al., 1983b; Larsson & Hagenfeldt, 1983). In addition, it has not proved possible to deplete cellular GSH levels below 20% of control levels, which may not be sufficiently low, to allow complete sensitisation. The problem of BSO toxicity could possibly be overcome by the use of rescue agents to quickly reverse the effects of the γ GCS inhibitor BSO (Anderson et al., 1985); glutathione esters could be used in this manner. Conversely, the higher level of intracellular GSH in tumours could be used to selectively activate pro drugs.

A major limitation in the effectiveness of cancer chemotherapy is the toxic effects to normal cells. Some compounds which contain sulphydryl groups are used clinically to reduce this effect. Mercaptoethane sulphonate (MESNA), for example, is used to protect the bladder against the cytotoxic side effects of cyclophosphamide and ifosfamide (Brock et al., 1982). The compound N-acetylcysteine has also been shown to be effective in this regard (Berrigan et al., 1982). This protection allows higher doses of the chemotherapeutic compounds to be given. Drug 'priming', which modulates GSH and GST's levels in the 'primed' normal cells, has also been used clinically as a strategy for protecting the bone marrow and intestine against high doses of melphalan. Initial clinical studies have also shown the usefulness of γ -L-glutamyl-L- Dopa (γ Gludopa) as a protective agent against the nephrotoxicity of cis platinum. This relies on the fact that γ GT activity is high in the kidney. γ GluDopa is actively absorbed into the kidney (Wilk et al., 1978), and metabolised to

dopamine which stimulates the dopaminergic response which, in turn, increases the rate of cis platinum excretion. High γ GT levels in tumours and in drug resistant models, as demonstrated here, compared with normal tissues (the kidney being the only exception), allows the prospect of a novel approach to chemotherapy using γ -glutamyl pro drugs to selectively target for tumours. Nephrotoxicity, which may be associated with this therapy, could be rescued with subsequent administration of γ GluDopa. Use of γ GT inhibitors to modulate tumour GSH levels may also be useful in sensitising tumours to chemotherapy.

GST's were involved in the adaptive response to cytotoxic drugs, but the changes observed were often paralleled with changes in GSH and other glutathione-dependent enzyme activities. The changes in GST expression in the drug resistant models, however, were not confined to one particular GST subgroup, although only certain GST's were elevated in response to a particular cytotoxic agent. As a consequence, expression of different GST subunits within a tumour may, therefore, modulate the response of the tumour to chemotherapy. Phenotyping the tumour for GST composition before giving chemotherapy, and even after the onset of drug resistance, may allow modification of the chemotherapy regime to optimise the response of the tumour to treatment. It may be possible to modify the response of the tumour to treatment by other means, such as pretreating the patient with high selenium, in order to selectively increase selenium-dependent GPX activity and decrease non-selenium dependent GPX activity before giving chlorambucil, a nitrogen mustard which may be detoxified by alpha class GST's (Robson et al, 1987; Buller et al, 1987). In addition, selenium deprivation may have the opposite effect, decreasing selenium-dependent GPX activity and increasing alpha class GST, allowing the treatment with adriamycin to have a greater effect.

Changes in GSH and glutathione-dependent enzymes may, therefore, be important in the modulation of the response of tumours to chemotherapy. These changes could occur as part of an adaptive response to cytotoxic drugs, as shown in the drug resistant models investigated in this project. It is hoped that this research, and the ideas put forward as a result, may contribute to the stimulation of future investigations into the role of glutathione and glutathione-dependent enzymes in tumour drug resistance, and, consequently, to an improvement in the effectiveness of cancer chemotherapy.

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APPENDIX

Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: intrinsic differences and cell cycle effects

D.Lewis¹, John D.Hayes² and C.Roland Wolf^{1,3}

¹Marshall Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, Department of Biochemistry, Hugh Robson Building, George Square, Edinburgh EH8 9XD. ²Department of Clinical Chemistry, University of Edinburgh, Edinburgh Royal Infirmary, Edinburgh EH3 9YW.

to whom reprint requests should be sent

The regulation of glutathione and various glutathione-dependent enzymes has been studied in two ovarian adenocarcinoma cell lines derived from a patient before (PE01) and after (PE04) the onset of drug resistance to *cis*-platinum, chlorambucil and 5-fluorouracil. Reduced glutathione levels were higher in the drug resistant cells (PE04). This could be attributed to a much higher (6.5-fold) γ -glutamyl-transpeptidase activity. In addition, glutathione-S-transferase (GST) and glutathione peroxidase were 2.9- and 2.3-fold higher in this cell line. Analysis of the GST subunit composition showed both cell lines contained high levels of the acidic and lower concentrations of a basic isozyme. The difference in GST activity between PE01 and PE04 did not appear to be related to the levels of these GST subunits. GSH, glutathione peroxidase and γ -glutamylcysteinyl synthetase were all found to be regulated during the cell cycle, higher levels being detected in logarithmic versus confluent cultures of PE01 and PE04 and MCF7. This did affect some of the differences between PE01 and PE04 and therefore may be a contributing factor to the differential sensitivity of these cells to cytotoxic compounds. The above data provide the first evidence that tumour cells obtained from a patient before and after the onset of drug resistance have significant differences in glutathione-dependent enzyme content.

Introduction

Acquired drug resistance represents a major problem in the treatment of various cancers which are initially responsive to therapy. Such cancers include small cell lung cancer, breast cancer and adenocarcinoma of the ovary. A variety of factors have been discussed in relation to this phenomenon. These include altered tumour vascularity and oxygenation, a selection for intrinsically resistant cells and drug-induced phenotypic changes which result in the over-expression of genes which confer resistance (1). In the latter case various mechanisms have been suggested including increased drug efflux (2), DNA repair (3) and detoxification (4).

Glutathione and glutathione-dependent enzymes are known to play a central role in drug detoxification (4-7). In relation to drug resistance, particular attention has been given to reduced levels of glutathione (GSH*) and the glutathione-S-transferases (GST)

(4,7-15). Indeed, there are several reports describing increased expression of GST and GSH in drug resistant cell lines (12-15). The GST are a multigene family of dimeric proteins which catalyse the conjugation of GSH to a wide variety of reactive electrophiles, including anti-cancer drugs (16). Characterization of the GST enzymes in human tissues has identified three distinct gene families of proteins which can be classified unambiguously by their subunit compositions as acidic, GST YfYf (subunit M_r 24 800), neutral, GST YbYb (subunit M_r 26 700) and basic, GST YaYa (subunit M_r 26 000) (17-19). In relation to drug resistance particular emphasis has been placed on the expression of the acidic GST which has been shown to be over-expressed in drug resistant breast cancer cells, as well as in experimental models such as preneoplasia (12,20). However, other GST subunits may also be important; for example, a CHO cell line made resistant to chlorambucil showed a marked elevation in the Ya/Yc subunits (15). Although the role of GST in conferring drug resistance in preneoplasia and cancer chemotherapy is circumstantial, there is strong evidence from other models that they are an important contributing factor (7).

In previous reports we have discussed the possibility that the altered expression of GSH and glutathione-dependent enzymes may represent a general protective response to cytotoxic insult (10,21). Other enzymes such as glutathione peroxidase (GPX) and glutathione reductase also protect cells against cytotoxic compounds, particularly those which generate toxic oxygen intermediates such as superoxides and peroxides (eg. adriamycin (22-24). The levels of these enzymes in tumour cells may also determine their susceptibility to a variety of cytotoxic drugs.

A significant problem in the study of drug resistance is establishing the relevance of *in vitro* experiments to clinical observations *in vivo*. In this study we have taken two ovarian adenocarcinoma cell lines, derived from a patient before (PE01) and after the onset of resistance (PE04) to a combination of *cis*-platinum, chlorambucil and 5-fluorouracil (25) to establish whether differences in GSH-dependent enzymes can be observed and therefore be a potential factor in the clinically observed acquired drug resistance.

Glutathione has been shown to be regulated during the cell cycle (26). In view of the altered sensitivity of cells in logarithmic growth to alkylating and chemotherapeutic agents (27,28) we have investigated the regulation of glutathione-dependent enzymes during the cell division to establish whether this may be a factor in the differential sensitivity to cytotoxic drugs.

Materials and methods

All chemicals were purchased from commercial sources and were of the highest grade of purity available.

Details of the derivation and general characterization of the cell lines PE01 and PE04 has been described previously (25). The PE04 cell line was more resistant to both *cis* platinum and chlorambucil and also showed cross resistance to a variety of other chemotherapeutic agents including adriamycin. No difference in the sensitivity of the lines to 5FU was observed. These ovarian cells, and the MCF-7 human breast cancer cell line, were cultured at 37°C, 100% humidity and 5% CO₂ in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS).

*Abbreviations: GSH, reduced glutathione; GST, glutathione-S-transferases; γ -glutamylcysteinyl synthetase; γ GT, γ -glutamyltranspeptidase; GPX, glutathione peroxidase; GRD, glutathione reductase; CDNB, 1-chloro-2,4-dinitro-5-aminobenzene; AMC, 7-amino-4-methyl coumarin.

Glutathione levels and glutathione-dependent enzyme activities in logarithmic and confluent cultures of PE01 and PE04

Thymidine incorporation	Activity					
	GSH	γ GCS	γ GT	GRD	GST	GPX
3805 \pm 387	27.8 \pm 6.7	16.8 \pm 0.64	0.06 \pm 0.01	62.3 \pm 5.8	74 \pm 9	11.6 \pm 0.7
12236 \pm 1232*	42.9 \pm 3.3**	23.1 \pm 5.6**	0.08 \pm 0.03	62.9 \pm 5.0	95 \pm 30	38.9 \pm 1.9*
29577 \pm 6174	37.4 \pm 5.2*†	12.2 \pm 0.8	0.39 \pm 0.04†	87.4 \pm 12.3	215 \pm 15†	27.0 \pm 0.8†
72682 \pm 12260*	77.7 \pm 19.4*	23.6 \pm 4.7*	0.33 \pm 0.07	89.5 \pm 16.2	213 \pm 39	52.1 \pm 2.1*

Thymidine incorporation is expressed as cpm per mg protein. GSH concentration is expressed as nmol/mg soluble protein. Enzyme activities are expressed as nmol/min/mg protein with the exception of γ GT which is expressed as nmol/min/10⁶ cells. Other details are given in Materials and methods section.

* and † are compared to PE01. * = significantly different from confluent culture. P < 0.001. † = significantly different from confluent culture. P < 0.01.

** = significantly different from PE01. P < 0.001.

† = significantly different from PE01. P < 0.01.

† = significantly different from PE01. P < 0.01.

† = significantly different from PE01. P < 0.01.

(100 μ g/ml) and penicillin (100 IU/ml). Insulin (2.5 mg/ml) was added to the cultures of PE01 and PE04.

Cells were harvested by trypsinisation and were always negative. Flasks were seeded at 2×10^5 or 5×10^4 cells/ml to confluent or logarithmic cultures respectively. Cell synchronisation was achieved by arresting the cells in G₀/G₁ of the cell cycle by reducing the concentration of fetal calf serum from 10% to 0.5% (29). Logarithmic growth was achieved by then increasing the FCS concentration to 10%. Cells were harvested later.

on

Cells were washed with 0.1% (w/v) trypsin, 0.001% (w/v) versene mix, washed with phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ pH 7.4) and resuspended at 1×10^7 cells/ml. Cell viability was determined to be greater than 95% as assessed by nigrosin dye exclusion. Cells were homogenised using a teflon glass, hand-held homogeniser.

Cells were lysed by sonication with three 5 s pulses at maximal power. A 5 s pulse at 4°C was included between each burst. The resulting sonicate was centrifuged for 20 min and the supernatant decanted. The supernatants were collected and stored at -70°C until required. GSH incorporation into DNA was measured with 0.19 MBq [³H]methyl-5-thioadenosine. Following incubation for 4 h the cells were washed with phosphate buffered saline, harvested and 50 μ l aliquots absorbed onto Millipore filters. Filters were then washed sequentially with distilled water, 5% (w/v) acid, distilled water and methanol. Filters were then dried, and measured by scintillation counting.

Enzymic assays

GSH was measured by high performance liquid chromatography following derivatisation with monobromobimane (Calbiochem, Cambridge Science, UK) (31). The stability of the GSH was established by the addition of 10% of GSH to a cell sample following harvesting. GSH in the supernatant was stable for up to 1 month if stored at -70°C. GST activities were determined using 1-chloro-2,4-dinitrobenzene and ethacrynic acid were determined by the method of Habig *et al.* (32). The peroxidase activity of the enzymes was determined by measuring the oxidation of cumene hydroperoxide as assessed by measuring the ADPH at 340 nm (33).

Glutathione reductase and glutathione peroxidase activities were assayed as described by Weiss *et al.* (34). γ -Glutamylcysteinyl synthetase activity was determined using a coupled reaction involving the measurement of NADPH at 340 nm (36). The particulate fraction of the cell preparation was assayed for γ GT activity (37) using γ -glutamyl-7-amino-4-methylcoumarin as substrate (Universal Biological Ltd, Cambridge, UK). All assays were carried out at 37°C. Statistical significance was determined using the Student's t-test.

Protein concentrations were determined by the method of Lowry *et al.* (38) using bovine serum albumin as standard.

Statistical analysis

Experiments were carried out using a modified version of that described by Adams *et al.* (39) as previously described by Adams *et al.* (40). Following SDS-PAGE and transfer to nitrocellulose, filters were probed for 10 min in 50 mM Tris-HCl buffer, pH 7.9 containing 0.05% (v/v) Tween 20 (TBST). Filters were blocked with TBST for 1 h followed by two 10 min washes with TBST. Specific binding was then added for 1 h, followed by four

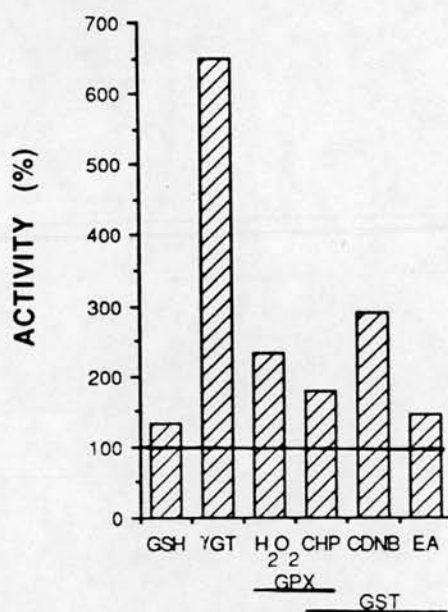


Fig. 1. Comparison of glutathione-dependent enzyme activities between PE01 and PE04 in confluent cultures. Experimental details are described in the Materials and methods section and in the legend to Table I. The PE04 values shown are expressed as a percentage of those obtained for PE01.

15 min washes with TBST. The filters were then incubated (1 h) with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase. Following further washes the bands were visualised using either 4-chloro-1-naphthol as peroxidase substrate or by autoradiography after labelling with 0.19 MBq [¹²⁵I]protein A.

Results

Measurements of GSH levels and glutathione-dependent enzymes, in drug sensitive (PE01) and resistant (PE04) cell lines, were initially carried out on confluent cultures (Table I, Figure 1) and in all cases where differences were observed the PE04 cell line had higher activities. Glutathione levels were significantly higher in this cell line (1.4-fold). Concomitant with the difference in GSH, γ GT activity was markedly elevated (6.3-fold). Increases in GST (2.9 fold) and glutathione peroxidase (2.3-fold) activities were also noted. The activities of glutathione reductase and γ GCS were essentially unchanged in both cell lines. In addition to the increase in GST activity towards the general substrate CDNB, the metabolism of ethacrynic acid, and cumene hydroperoxide was also significantly higher. The activities being 40.0 ± 6.8 and 58.4 ± 3.4 nmol/min/mg for ethacrynic acid and

Fig 2

G031-88

70% ~~base~~ ^{HR}

Glutathione S-transferase and isozyme expression in cell lines PE01 and PE04. 50 μ g of supernatant protein was subjected to SDS-PAGE, transferred to nitrocellulose and probed with antibodies to the acidic (YfYf, basic (B₁B₁)) GST isozymes. Blots were developed using I¹²⁵. Protein details are described in Materials and methods section.

± 5.4 and 26.1 ± 8.1 nmol/min/mg for cumene hydroperoxide for PE01 and PE04 respectively. Western blots were carried out to establish the GST subunit composition of the two ovarian adenocarcinoma cell lines and determine whether the difference in GST activity towards β -ethacrynic acid and cumene hydroperoxide could be related with differences in the expression of known GST isozymes (Figure 2). Both cell lines had high levels of the acidic subunit. The basic Ya subunit could also be detected and was present at approximately 1/10th of the concentration of the acidic subunit. The neutral Yb GST subunit could not be detected in either cell line. The content of the acid Ya-type subunits was similar in both cell lines, although the level of the Ya subunit did appear marginally higher in PE04. These data indicate that the levels of these subunits are not responsible for the large difference in GST enzymic activity. Glutathione concentration has been shown to change during the cell cycle (26). We therefore investigated which of the glutathione-dependent enzymes were also subject to such variation, particularly as they could contribute to the differences in the cell lines and also be a factor in their sensitivity to cytotoxic drugs. A 2- to 3-fold increase in tritiated thymidine incorporation in both cell lines synchronized in logarithmic growth was observed (Table I, Figure 3). A large difference in thymidine incorporation between PE01 and PE04, indicating a higher rate of DNA synthesis in PE04, this could be due to differences in thymidine kinase activity or other enzymes involved in DNA synthesis. Glutathione concentration was significantly elevated in both cell lines in logarithmic growth (Table I, Figure 3). The increase was 1.6- and 2.1-fold, relative to confluent cultures, for PE01 and PE04 respectively. In cultures in logarithmic growth the GSH level of PE04 was 1.8-fold higher than an equivalent PE01. A significant increase in glutathione concentration was also observed for the MCF-7 cell line during logarithmic growth from 34.2 ± 17.4 to 64.7 ± 17.0 nmol/mg protein. Concomitant with the increase in glutathione levels to 2-fold increase in γ GCS activity was observed. The

enzymic activity being increased 1.8-fold in the MCF-7 cell line from 7.1 ± 2.3 to 13.0 ± 3.7 nmol/min/mg protein.

Glutathione peroxidase activity using hydrogen peroxide as substrate was also significantly elevated (1.9- and 3.4-fold) in dividing cells in all three cell lines (Figure 3). GPX activity being increased in the MCF-7 cell line from 34.1 ± 10.1 nmol GSH oxidised/min/mg protein to 76.8 ± 21.7 nmol GSH oxidised/min/mg protein. The fold elevation was higher in the PE01 than in PE04 (Table I, Figure 3). The GST activity was not significantly changed during the cell cycle.

Discussion

The two ovarian cell lines used in this study represent a valuable *in vivo* model for the study of drug resistance (25). Karyotype analysis showed that the cell lines were of the same clonal origin but that they diverged from each other either before, or during, drug treatment. Although it is not clear how representative the cell lines are of the original tumour, the data in this paper provide evidence that the tumour cells isolated from a patient who had become resistant to drug therapy contained higher levels of glutathione and a variety of glutathione-dependent enzymes than those obtained from the same individual prior to the onset of drug resistance. It is not clear whether the increased enzyme levels in the resistant cell line was an intrinsic property of these cells or was a consequence of the drug treatment. There is however evidence which suggests that glutathione and GSH-dependent enzymes increase as a result of chemical stress (4,11,21,42).

There is a growing body of evidence which indicates that the glutathione content is an important factor in the susceptibility of tumour cells to a range of distinct cytotoxic drugs. Resistance to *cis*-platinum provides a good example of the relationship between drug susceptibility and GSH levels (43-45). There is strong evidence that glutathione plays an important role in protection against alkylating agents. This is particularly the case for ovarian cancer where Ozols and co-workers have demonstrated that glutathione depletion will sensitize cells to the toxic effects of melphalan, *cis*-platinum and adriamycin (46,47). In addition, there are now several reports which demonstrate that cell lines made resistant to alkylating agents or *cis*-platinum have increased glutathione and GST activity (48). On the basis of the current study, the elevation in glutathione content in PE04 may be a direct consequence of a very substantial increase in γ GT activity. This enzyme has been implicated in the regulation of cellular levels of this cofactor (49-51). It is noteworthy that γ GT is elevated in rat liver preneoplastic foci which also have elevated GSH levels. It has recently been shown that in mouse lymphoma L1210 cells made resistant to L-phenylalanine mustard (L-PAM), a close correlation between γ GT levels and levels of GSH was observed (50). It is worthy of note that glutathione reductase, which is also involved in determining reduced GSH levels, was not elevated in the drug resistant PE04 line.

A substantial difference between the cell lines was observed in GST activity which was not directly attributable to expression of any of the isoenzymes studied. Whether novel forms of these enzymes are present in ovarian tissue or whether the Western blots do not reflect the active enzyme concentration is at present unclear. In recent studies we have identified acidic and basic GST subunits as major GSTs in primary ovarian tumours (Lewis *et al.*, unpublished). There is an increasing body of evidence indicating that the expression of these proteins within tumours will be an important factor in their sensitivity to anticancer drugs such as melphalan (7,16,1), cyclophosphamide (11,14) and probably chlorambucil (15). Higher GST levels in the PE04 cell line may

contribute to the differences in drug sensitivity observed. It is interesting that both cell lines contained high levels of the acidic subunit (sometimes known as γ) which has often been discussed in relation to the effect of cells to toxic compounds (7,12,42).

In addition to the GST enzymes several other glutathione-dependent enzymes including glutathione peroxidase are involved in the detoxification of cytotoxic chemicals (52). Many of these are only studied in relation to drug resistance. It was interesting that glutathione peroxidase levels were found to be significantly higher in the PE04 cell line. This enzyme has a role in the reduction of organic and lipid hydroperoxides. Such intermediates have been implicated in the mechanism of action of a variety of anticancer drugs (9,10). The concentration and the activity of γ -GCS and glutathione peroxidase were elevated in dividing cells. These were observed in both ovarian cell lines and the MCF7. These experiments were carried out to determine the effect of cell cycle effects on these enzymes could be a factor in drug sensitivity. PE01 and PE04 were affected in a similar manner although the extent of the cell cycle effects was different. This could play a role in the relative sensitivity of the cell lines to cytotoxic drugs. Cell cycle effects in the effects of cytotoxic drugs and carcinogens are well documented (27,28) and the over-expression of GSH could increase resistance of dividing cells to certain drugs (53,54). The increase in GSH concentration in the PE04 may be a direct consequence of the increased γ -GCS. The cell cycle effects observed here exemplify the need for controlled conditions when measuring and comparing drug sensitivity and related enzymes in cell culture.

Further evidence is presented demonstrating higher levels of glutathione and glutathione-dependent enzymes in a tumour cell line derived from a patient following treatment of resistance to cytotoxic drugs. It is perhaps of interest that a variety of glutathione-dependent enzymes, including the glutathione transferases, were also elevated. These may also play an important role in intrinsic and acquired drug resistance.

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Classification: Cell Biology

**AMPLIFICATION AND INCREASED EXPRESSION OF ALPHA CLASS
GLUTATHIONE S-TRANSFERASE GENES ASSOCIATED WITH RESISTANCE TO
NITROGEN MUSTARDS.**

(Drug Resistance/Glutathione/Preneoplasia/Cancer Chemotherapy)

Alex D. Lewis*, Ian D. Hickson[†], Craig N. Robson[†], Adrian L.
Harris[†], John D. Hayes[‡], S.A. Griffith[§], M.M. Manson[§], A.E. Hall*,
J.E. Moss* and C. Roland Wolf*[¶]

* Imperial Cancer Research Fund
Laboratory of Molecular Pharmacology and Drug Metabolism
University Department of Biochemistry
Hugh Robson Building
George Square
Edinburgh EH8 9XD
Scotland, UK

[†] Department of Clinical Oncology
University of Newcastle-upon-Tyne
Royal Victoria Infirmary
Newcastle-upon-Tyne NE1 4LP
England, UK

[‡] Department of Clinical Chemistry
University of Edinburgh
Edinburgh Royal Infirmary
Edinburgh EH3 9YW
Scotland, UK

[§] MRC Toxicology Unit
Woodmansterne Road
Carshalton
Surrey SM5 4EF
England, UK

[¶] Author for correspondence

ABSTRACT

Glutathione dependent enzymes play a central role in the protection of cells from cytotoxic chemicals and have been implicated in the intrinsic and acquired resistance of tumors to cytotoxic drugs. We have generated a CHO line resistant to bifunctional nitrogen mustards and in this report have characterized and isolated the protein which represents the major observable phenotypic difference between the drug sensitive and resistant cell lines. This purified protein is shown to be an alpha class glutathione S-transferase comprising YcYc subunits and possessing a pI value of approximately 8.0. The intracellular level of the Yc subunit is elevated greater than 40-fold in the drug resistant cell line which could account for the increase in glutathione S-transferase activity towards both 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide. Other glutathione S-transferase subunits within this gene family are also elevated. Southern analysis indicates that the genes coding for these proteins are amplified 4-8 fold in the drug resistant cell line. In addition, γ -glutamyltranspeptidase activity is increased 3.6-fold in the drug resistant CHO cell line, which may explain the increase in cellular glutathione level.

The above data indicates that gene amplification may play an important role in drug resistance towards alkylating agents and also that other enzymes in glutathione homeostasis are involved. These observations underline the strong similarities between drug resistance and the phenotypic changes which occur in chemical-induced neoplasia.

Abbreviations: CHO, Chinese Hamster Ovary; GST, Glutathione S-transferase; GSH, Reduced glutathione; γ GT, γ Glutamyl-transpeptidase;

INTRODUCTION

The ability to resist the chemical stresses imposed by our environment represents a major evolutionary driving force. It is clear that a wide variety of defense systems have evolved to protect against such cytotoxic insults. Studies into tumor cell drug resistance and carcinogenesis have greatly increased our understanding of these systems and various defense mechanisms appear to be important including, membrane permeability (1), DNA repair (2), gene amplification (3), and drug detoxification (4).

For several decades glutathione and glutathione-dependent enzymes have been implicated in drug detoxification reactions (5). Increased glutathione content, resulting in increased drug detoxification, was proposed as a mechanism of drug resistance in tumor cells over twenty years ago (6, 7). However, it is only recently that these observations have become a major topic of study. There is now substantial evidence which indicates that the overexpression of glutathione and glutathione-dependent enzymes is an important mechanism of acquired drug resistance in both normal and tumor cells (4-13).

In a previous report we described a chlorambucil-resistant CHO cell line, also cross resistant to other bifunctional nitrogen mustards (14). These cells have elevated GSH and glutathione S-transferase (GST) activity but do not exhibit differences in drug accumulation or DNA repair (15). The major observable phenotypic difference between the sensitive and resistant cell line is in the expression of a protein of molecular weight approximately 25000. Here we report the isolation and characterization of this protein which is shown to be a GST whose overexpression results from gene amplification. In addition, we also show that other glutathione S-transferase subunits and

γ -glutamyltranspeptidase (γ GT) are elevated in the resistant line.

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest grade available.

Cell Culture: The two cell lines used in this study have been termed CHO-K1 (wild type) and CHO-Chl^r. The latter cell line exhibits a 24-, 34- and 14-fold higher resistance to chlorambucil, mechlorethamine and melphalan, respectively (14). Cells were maintained in Ham's F10 medium supplemented with 5% fetal calf (and in some cases 5% newborn calf) serum (v:v), streptomycin (100 µg/ml) and penicillin (100 IU/ml) and cultured in 100% humidity and 5% CO₂ as monolayers at 37°C.

Isolation of Subcellular Fractions: Cells were harvested from confluent cultures with 0.1% (w:v) trypsin and 0.001% (w:v) versene, washed three times in phosphate buffered saline (140 mM NaCl/ 2.7 mM KCl/ 8 mM NaPO₄/ 1.5 mM NaPO₄ pH 7.4; PBS) and resuspended in 400 µl of this buffer. Samples were then sonicated with three 5 sec pulses at maximal power with a 5 sec cooling period at 4°C between each step. The resulting sonicate was centrifuged at 18,000 g for 20 min and the supernatant decanted. Protein was estimated in freshly prepared cell fractions by the method of Lowry et al (16), using bovine serum albumin as the standard. Cell fractions were stored at -70°C before further biochemical analyses were undertaken; samples were stable under the storage conditions used.

Enzymic assays: GST activity towards the substrates 1-chloro-2,4-dinitrobenzene, ethacrynic acid and cumene hydroperoxide were determined in the supernatant fractions by the methods of Habig et al (17) and Stockman et al (18). Glutathione reductase was assayed fluorometrically, as described by Weiss et al (19), and glutathione peroxidase activity determined

spectrophotometrically (20). The activity of γ -glutamyl cysteinyl synthetase, the rate determining enzyme of GSH biosynthesis, was determined using a coupled reaction by measuring the rate of oxidation of NADH spectrophotometrically (21). The particulate cell fraction of the cell preparation was taken for the assay of γ GT using γ -L-glutamyl-7-amino-4-methyl coumarin as substrate (Universal Biologicals Limited, Cambridge, UK) (22).

Western Blot Analysis: SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (23). Western blots were carried out using essentially the method described by Towbin *et al* (24), as modified by Adams *et al* (25). The antibodies employed were raised against the human pi class GST (λ , YfYf subunits), human alpha class GST (B_1B_1 , YaYa subunits), and human mu class GST (μ , YbYb subunits). In addition, antisera to the rat alpha class GST, YaYa or YcYc subunits were also used. Antisera were prepared as described previously (26,27).

Two-dimensional electrophoresis was carried out according to the method of O'Farrell (28) as modified by Robson *et al* (14). A pH 5-8 gradient was employed in the isoelectric focussing step which represented the first dimension. Either 100 μ g of total cellular protein or 5 μ g of purified protein was loaded for each experiment.

Purification of Glutathione S-transferases: Cells from the CHO-Chl^r (5×10^9 cells), were harvested, frozen at -70°C and lysed as described by Soma *et al* (29). Briefly, the pellets were thawed and suspended at 4°C in 40 mM Tris/HCl buffer, pH 7.4, containing 160 mM KCl, 4 mM EDTA and 5 mM dithiothreitol. The final volume was 5 ml. The suspension was homogenized with a teflon/glass homogenizer (25 strokes), centrifuged for 30 sec in an Eppendorf Microfuge, and then centrifuged for a further 45 min at 35,000 rpm. The resulting supernatant was applied to a column of

S-hexylglutathione (4 ml bed volume) equilibrated with 10 mM Tris/HCl, pH 7.8, containing 0.2 M NaCl and 3 mM 2-mercaptoethanol. The column was washed with the above buffer and the GST eluted by the addition of 5 mM S-hexylglutathione to the running buffer. Using this procedure the majority of the GST in the cell lysate were retarded by the affinity gel but eluted in the wash fraction from the column. The enzyme-containing fractions were combined, dialysed against 20 mM Tris/HCl, pH 7.8, 1 mM EDTA and 5 mM mercaptoethanol and reapplied to the S-hexylglutathione-Sepharose column. The GST, which was now retained by the affinity matrix following this second chromatographic step, was then eluted with a solution of 5 mM S-hexylglutathione in the 20 mM Tris/HCl running buffer, pH 7.8. Two proteins were detected in the eluate, a minor component which eluted immediately and a major protein with GST activity. This protein was of high purity as judged by SDS-polyacrylamide-gel electrophoresis (see Results section).

DNA and RNA Analysis

A full length cDNA clone alpha class GST (pMP 37, 942bp) was isolated from a human liver λ gt11 library using an oligonucleotide coding for the six NH₂-terminal amino acids of the B₁B₁ protein. Over the coding region this cDNA had an identical sequence to that published by Board & Webb (30). A full length rat liver γ -glutamyl transpeptidase cDNA clone was isolated from a cDNA library in λ GT10 using a cDNA to the rat kidney enzyme (31). Eco RI digest of this clone yielded two fragments. The 5' fragment (pEGL1.1) of 1230bp was used as a probe.

DNA Isolation

10⁷ cells were washed twice with phosphate buffered saline (PBS) and then harvested by scraping into PBS. Cells were then lysed in 0.5% SDS, 150mM NaCl, 10mM EDTA, 10mM Tris-HCl pH7.5

(2mls) and treated with RNase A (100 μ g/ml) then followed by proteinase K (250 μ g/ml) at 37°C for 1hr and 4hr respectively. Samples were then extracted with phenol followed by phenol/chloroform (1:1 v/v) and the DNA precipitated in 60% EtOH containing ammonium acetate (1M). The precipitated DNA was wound onto a glass rod, air dried and resuspended in 10mM Tris-HCl pH7.5, 1mM EDTA. DNA concentrations was determined from the absorption at 260nm. Southern analysis was carried out as described by Hill et al (32).

RESULTS AND DISCUSSION

The levels of a variety of glutathione-dependent enzymes in the drug sensitive and resistant CHO cell lines are shown in Table 1. In agreement with a previous report, both the GSH level and the GST activity (towards CDNB) were significantly higher in the chlorambucil resistant cell line (1.8- and 2.7-fold, respectively). In addition, the peroxidase activity towards cumene hydroperoxide was also elevated 5.1-fold. This increase appeared to be completely due to differences in GST expression as the selenium-dependent activity (determined using H_2O_2 as substrate) was similar in both cell lines (Table 1). The majority of the peroxidase activity towards cumene hydroperoxide in the CHO-KI was mediated by the selenium-dependent enzyme. When this contribution was subtracted from the values obtained, CHO-Chl^r had approximately 50-fold higher GST-mediated peroxidase activity than the wild-type cell line.

A variety of enzymes are involved in maintaining reduced glutathione levels within cells (33). Three of these; glutathione reductase, γ -glutamyltranspeptidase and γ -glutamyl cysteinyl synthetase were measured. Only γ GT was elevated significantly in the drug resistant cell line, suggesting that this protein may be responsible for maintaining the elevated cellular GSH level. Elevated γ GT concentrations have been observed in cell lines resistant to cytotoxic drugs (34,35). In addition, the fact that preneoplastic foci in rat liver, which have elevated GSH content, also have elevated γ GT levels (36), supports the hypothesis that this enzyme is involved in maintaining increased cellular GSH levels. It is interesting that in the models for drug resistance induced by alkylating agents we have studied, γ GT activity is consistently elevated (35, and unpublished).

Our initial studies indicated that altered expression of the alpha class GST represented the major change in GST expression in the CHO-Chl^r cell line (15). In support of this the staining pattern of cytosolic samples clearly showed an elevation in a protein with a mobility similar to that of the rat Yc GST subunit (Figure 1). This subunit could therefore be the polypeptide, with an estimated Mr of 25000, which was shown previously to be dramatically overexpressed in the CHO-Chl^r cell line (15); more recent work has estimated the Mr of the Yc subunit to be 27000-27500 (37).

In order to establish whether this was the case the overexpressed protein was purified. In addition Western blots of CHO cytosol with antibodies to known rat or human GST subunits (Ya, Yb, Yc and Yf) were carried out. A two step purification procedure (Figure 2) yielded a pure protein of similar mobility to the rat Yc (Mr 27500) standard. This protein was shown to be a GST based on its activity towards CDNB, cumene hydroperoxide and ethacrynic acid, the turnover numbers for these substrates being 6031, 964 and 550 nmol/min/mg protein, respectively. The relative activities of the Chinese hamster GST towards these three substrates is similar to that exhibited by the rat Yc subunit (38). Western blots showed that the purified protein and the protein overexpressed in CHO-Chl^r reacted strongly with antibodies to the alpha class Yc GST subunit (Figure 3). This blot also demonstrated a marked 40 fold difference in the expression of this protein between the drug sensitive and resistant cell lines. In order to confirm these findings the mobility of the purified protein following electrophoresis in two-dimensional polyacrylamide gels was compared with the over expressed protein in the CHO-Chl^r cells, following two-dimensional electrophoresis. The two proteins were found to have identical mobilities in this

system (Figure 4). The isoelectric point of the purified protein being approximately pH 8.0.

In view of the observation that other GST subunits may be involved in drug resistance Western blots were carried out with antibodies to other GST enzymes (Ya, Yb and Yf) (Figure 5). Slight differences between CHO-K1 and CHO-Chl^r in the expression of the pi and mu class proteins were observed however these differences were marginal. However, a significant elevation in Ya, another alpha class subunit, was observed. This antibody also cross-reacted weakly with the Yc subunit (Figure 5).

In order to establish the molecular mechanism for the over expression of alpha class GST. DNA from the cell lines was analyzed by Southern blot analysis (Figure 6). The complexity of the banding pattern obtained is indicative of a multigene family incorporating the Ya and Yc genes. In Chinese hamsters this gene family appears to cover up to 100kb of DNA. The intensity of most of the bands was much higher for CHO-Chl^r than the wild type CHO-K1, and based limited dilution of CHO-Chl^r DNA until the bands were of equal intensity to CHO-K1 it appeared that there was a 4-8 fold elevation in gene copy number (not shown). Densitometric scanning of the bands indicated that the increase may be up to twenty fold. This elevation was however not uniform and some bands were essentially unchanged. No difference between CHO-K1 and CHO-Chl^r was observed when a γ -glutamyltranspeptidase cDNA was used as a probe (Figure 7B).

The above data provides strong evidence that the class alpha GST are the major proteins over expressed in CHO cells made resistant to chlorambucil. This increase in expression appears to be a consequence of a gene amplification event. It is worthy of note that DNA repair capacity and also drug accumulation are unchanged in the CHO-Chl^r cells (15) and that the mechanism of the

drug resistance appears to be due to enhanced drug detoxification which results in reduced DNA damage (15). The alpha class GST subunits therefore appear to be important in this mechanism. In support of this possibility GST's have been shown to catalyze the conjugation of melphalan to GSH, leading to its detoxification (39). Melphalan is a structural homologue of chlorambucil to which CHO-CH1^r is also resistant. Which GST subunit is responsible for this reaction however has not been established.

The molecular mechanisms responsible for the changes in the levels of GST and other glutathione-dependent enzymes have remained elusive. However, we have now obtained evidence that gene amplification may be an important part of this process, and therefore may play an important role in drug resistance induced by alkylating agents. The time point at which this amplification occurs ie. whether some cells intrinsically contain amplified sequences of whether gene amplification is induced by chemical exposure is unclear.

The phenotypic changes observed in protein expression in drug resistance, ie. in glutathione-dependent enzymes, P-glycoprotein and DNA repair enzymes bear a remarkable similarity to those observed in chemical-induced neoplasia in rat liver (12,40-42). These changes are often independent of the chemical reagent used and may well reflect the existence of a fundamental cellular response mechanism to combat chemical insult. Whether common genetic changes are involved in such a response is at present unknown.

The role of glutathione-dependent enzymes in this adaptive change is uncertain, however, the ubiquitous nature of the changes observed in the expression of these proteins following cytotoxic insult indicates that they play an important role. For example, changes in levels of these enzymes have been reported to occur

during oxidative stress in both bacteria (43) and rodent lung (44) and also in chemical-induced stress in the mouse bone marrow (13, 45). With particular regard to the GST it is known that in preneoplasia in rat liver the Ya, Yb, Yc and Yf GST subunits are overexpressed (12,40,41,46,47). The Yf subunit has also been shown to be overexpressed in MCF7 cell lines made resistant to adriamycin (48) or to a variety of other chemotherapeutic agents (Hill et al, unpublished). The present study demonstrates that in tumor cell drug resistance the expression of other GST subunits as well as a variety of glutathione-dependent enzymes, could be of significant importance in the resistance mechanism. Over expression of these genes, for example, as a consequence of a gene amplification, implies the presence of another form of the multidrug resistance phenotype involving alkylating agents and compounds which are themselves, or which generate, peroxides.

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FIGURE 1: Protein Staining pattern of CHO-K1 and CHO-Chl^r

Samples were separated by SDS-PAGE. 50 µg of soluble protein was taken per track. The mobilities of the rat Yc, Yb and Yf standards are also shown. K1 and Chl^r are the wild type (S) and resistant (R) cell lines, respectively.

FIGURE 2: Purification of the overproduced protein of molecular weight 27500 from CHO-Chl^r cells

Samples were taken at various stages during the purification and run on 11% SDS-PAGE gels and stained with Coomassie Blue. Tracks 1,2,3, and 4 are respectively total cell extract, peak of GST activity eluted from the S-hexylglutathione column, column flow through in the presence of 0.2 M salt and the purified GST obtained following rechromatography of sample in track 3 respectively.

FIGURE 3: Comparison of glutathione S-transferase subunit content in CHO-K1 and CHO-Chl^r and the purified protein

Western blots were carried out as described in the Materials and Methods section. Yc = standard; K1 and Chl^r are samples from the sensitive and resistant cell lines; P is the purified GST protein (5µg). The antibody used was to the rat Yc subunit.

FIGURE 4: Two Dimensional Gel Electrophoresis of CHO-Chl^r and the Purified GST Protein

Gels were run as described in the Materials and Methods and in reference 14. 100 µg of soluble protein from CHO Chl^r (top panel) or 5 µg of the purified GST enzyme (bottom panel) were taken. The overexpressed protein in CHO-Chl^r is indicated by a curved arrow.

FIGURE 5: Glutathione S-transferase Subunit Content in CHO-K1 and CHO-Chl^r

Western blots were carried out as described in the Materials and Methods sections using 50 µg of soluble protein per track.

The bands were identified using the human antibodies to the Yf, class pi; Ya, class alpha; and Yb, class mu GST subunits; STD = Human GST standard (1 µg); K1 = CHO-K1; Chl^r = CHO-Chl^r.

FIGURE 6: DNA analysis of CHO-K1 and CHO-Chl^r using an Alpha Class GST and γGT cDNA's as Probes

Two DNA samples from separate cultures for each cell line were digested with restriction endonucleases according to the manufacturers instructions. (Tracks 1 and 2 - CHO-K1, 3 and 4 - CHO-CHL^r). DNA (5µg) was separated on a 1% agarose gel and transferred to Hybond-N. Fragments hybridizing the human alpha class GST cDNA (A) or the γ-glutamyl transpeptidase cDNA (B) were detected as described by Hill *et al* (32). On the basis of absorption at 260nm 1.4 fold more DNA was loaded in track 4. Molecular sizes in kilobases are given on the right hand side.

TABLE 1 **Glutathione-dependent enzyme expression in CHO-K1 and CHO-Ch1^r Cell Lines.**

Cell line	Activity							
	GSH	GST			GPX	γGT	γGCS	GRD
		CDNB	EA	CHP				
CHO-K1	18.4±2.2	239±36	19.7±3.2	16.4±2.0	8.9±4.8	0.82±0.26	70.0±16.2	96.5±23
CHO-Ch1 ^r	33.0±5.6	638±115 ⁺	38.0±12.1 [†]	83.4±18.3*	10.0±2.7	2.85±0.48*	97.7±51.8	129.5±61.5

The abbreviations used are: GSH, glutathione; GST, glutathione S-transferase; GPX, glutathione peroxidase;

γGT, glutamyltranspeptidase; γGCS glutamyl cysteinyl synthetase; GRD, glutathione reductase; CDBN, 1-chloro-2,4-

dinitrobenzene; EA, ethacrynic acid ; CHP cumene hydroperoxide. All reactions were carried out at 37°C. Values are expressed

as nmoles/min/mg protein; for CDBN, CHP, EA, H₂O₂, γGCS and GRD or nmoles/min/10⁶ cells for γGT. GSH content is expressed as

nmol/mg protein.

* p < 0.005., ⁺ p < 0.01., [†] p < 0.07.

Induction and Suppression of Glutathione Transferases by Interferon in the Mouse*

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David J. Adams^{‡§}, Franny R. Balkwill[¶], Dorotka B. Griffin[¶], John D. Hayes^{||**}, Alex D. Lewis[‡], and C. Roland Wolf^{‡‡}

From the [‡]Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, University Department of Biochemistry, Hugh Robson Building, George Square, Edinburgh, United Kingdom, the [¶]Imperial Cancer Research Fund, Interferon Laboratory, Lincoln's Inn Fields, London, United Kingdom, and the ^{**}Department of Clinical Chemistry, University of Edinburgh, Royal Infirmary, Edinburgh, United Kingdom.

The administration of interferon- α/β to female nude (nu/nu) mice caused significant changes in the levels of the cytosolic hepatic glutathione transferases. Antibodies raised against rat subunits, Ya, Yc, Yb₁, Yb₂, and Yk, and the subunits of the human transferases, μ (Y_b Y_b), λ (Y_r Y_r), and ϵ (B₁ B₁) all reacted with enzymes in the mouse and were used to demonstrate suppression and induction of transferase levels. Western blot analysis followed by semiquantitation by laser scanning showed the Ya, Yb₁, Yb₂, Yc, Yk, μ , and B₁ subunits to be suppressed by 11, 11, 44, 30, 12, 14, and 47%, respectively, by interferon treatment. In contrast to these findings, the Yf subunit was induced 5–7-fold. A concomitant 220% increase was observed in the specific activity of the hepatic cytosol for ethacrynic acid, a substrate for the Yf subunit. Changes in the levels of transferase enzymes in normal and tumor cells may have significant implications when cytotoxic drugs are used in combination with interferons in cancer therapy. The Yf subunit, an enzyme found in human tumors and in placenta (Polidoro, G., Di Mio, C., Del Boccio, G., Zulli, P., and Fererici, G. (1980) *Biochem. Pharmacol.* 29, 1677–1680) has also been shown to be elevated in hepatic preneoplastic lesions (Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H., and Ito, N. (1984) *Cancer Res.* 44, 2698–2703). These data indicate that the Yf subunit represents a potentially important interferon-inducible gene product.

The glutathione transferases play a central role in the protection of cells from toxic and carcinogenic electrophiles, as well as from reactive oxygen intermediates such as superoxide and peroxides (1). In some cases they have also been implicated in the activation of chemical carcinogens (2, 3). These cytosolic enzymes are dimeric proteins coded by multiple genes which are part of four or more clearly defined gene families. They are predominantly localized in the liver but have been detected in a large number of other tissues (4–8). The relative distribution and regulation of these isozymes

within both normal and tumor cells will be an important factor in their susceptibility to toxins and carcinogens (9). Our interest in the regulation of drug-metabolizing enzymes by interferons stems from (a) the potential effects of viral infection on the metabolism and disposition of drugs and carcinogens and (b) the potential use of interferons in conjunction with chemotherapeutic agents, which are metabolized by these enzymes, in the treatment of cancer (10). Interferons have anticancer activity in man, but this has only been of therapeutic benefit in cancers such as hairy cell leukemia, low grade lymphoma, and chronic myelogenous leukemia (11). However, in animal models, when used in conjunction with alkylating agents, or compounds such as adriamycin, they cause a significant enhancement in antitumor activity (10–14). The underlying mechanism of this effect is unclear, although a recent report suggested that the increased activity of combination therapy was due to effects on the tumor rather than on host drug-metabolizing enzymes (15). However, it has been clearly shown that significant changes occur in both the hepatic cytochrome P-450 enzyme system and glutathione transferase activities when interferon is injected into mice (15, 16). Such changes may be of significant importance in drug disposition and toxic side effects when interferon-drug combinations are used in man.

The elevation of glutathione transferases in preneoplastic nodules is thought to be an important aspect of chemical-induced neoplasia (17) as well as in the acquired resistance of tumor cells to chemotherapeutic agents (18). Salerno *et al.* (19) have reported that administration of interferon in conjunction with 3-methylcholanthrene significantly reduced the incidence of liver tumors in mice. This could be due to effects on cytochrome P-450 or glutathione transferase expression. For the above reasons we have characterized interferon-induced changes in the expression of particular glutathione transferase enzymes.

MATERIALS AND METHODS

Female 6-week-old BALB/c or nude (nu/nu) mice were used. Nude mice were housed in negative pressure isolators and were specific pathogen-free. Experiments were carried out on these animals because they were those used in xenograft experiments described in previous publications (10, 15). The nude mice were treated daily for 30 days (BALB/c mice for 3 days) with 2×10^5 μ m of mouse EA cell interferon kindly supplied by Dr. S. Moushowitz, Enzo Biochem, New York (specific activity 5×10^7 μ m/mg) or mouse C243 cell interferon kindly supplied by Dr. E. Proietti, Istituto di Superiore Sanita, Rome (specific activity 2×10^6 μ m/mg). Interferon injections were given subcutaneously. The peak serum levels using this dose were 200 μ m/ml 2 h after injection. Interferons were assayed in a biological assay that measured reduction in viral RNA synthesis and were calibrated against mouse reference standard g 002-904-511 (National

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§ Present address: Dept. of Microbiology, University of Leeds, Leeds, UK.

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‡‡ To whom correspondence should be addressed.

TABLE I

Effect of interferon on glutathione transferase activity

Mouse cytosol fractions were prepared from groups of three animals treated with interferon. Enzymic assays were carried out at 37 °C as described under "Materials and Methods." Values are expressed as μmol of product/min/mg of cytosolic protein and are given as mean \pm S.D. for three determinations.

	Enzyme activity						
	Ethacrynic acid	1-Chloro-2,4-dinitrobenzene	<i>p</i> -Nitrophenyl acetate	<i>t</i> -4-Phenyl-3-buten-2-one	2,4-Dichloronitrobenzene	Bromosulphophthalein	1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane
	$\mu\text{mol/min/mg protein}$						
Control	0.077 \pm 0.016	7.00 \pm 0.50	0.20 \pm 0.03	0.0011 \pm 0.0005	0.151 \pm 0.017	0.013 \pm 0.002	0.068 \pm 0.018
Interferon	0.169 \pm 0.014	7.45 \pm 1.27	0.23 \pm 0.04	0.0009 \pm 0.0002	0.117 \pm 0.006	0.010 \pm 0.005	0.078 \pm 0.010

Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Hepatic cytosol fractions were prepared as described previously (15). Samples were stored at -70°C before use. Protein determinations were by the method of Lowry *et al.* (20) using bovine serum albumin as standard. Glutathione transferase activity toward the substrates 1-chloro-2,4-dinitrobenzene, ethacrynic acid, *p*-nitrophenyl acetate, *t*-4-phenyl-3-buten-2-one, bromosulphophthalein, and 1,2-epoxy-3-(*p*-nitrophenoxy)propane was carried out at 37°C by the methods described by Habig and Jakoby (21). Glutathione transferases¹ L (Ya Ya), A (Yb₁ Yb₁), D (Yb₂ Yb₂), AA (Yc Yc), and K (Yk Yk) were purified from rat liver and human transferases ϵ (B₁ B₁), μ (Yb Yb), and λ (Yf Yf) from human liver or lung by methods described previously (22–25). Antibodies against these proteins were raised in rabbits and used for the "Western blot" procedure at a dilution of 1:1,000. The antibodies used have been shown to react with autologous proteins in the mouse (26). The Ya, Yb₂, Yc, Yk, and Yf antibodies used in this study were specific for their autologous antigens (8). However, there was some reactivity between the Yb₁ antibody and Yb₂, between μ and Yb₂, and between the human basic B₁ enzyme and Ya (8). Immunoprecipitation experiments were carried out by incubating 100 μl of hepatic cytosol with varying amounts of λ antiserum (1–100 μl) for 12 h at 4°C . The volume was always made up with preimmune serum. The control sample contained preimmune serum only. After this period anti-rabbit IgG was added (30 μl /30 μl sample) and after 3 h at 4°C the sample spun at 13,000 rpm for 5 min. The clear supernatant was taken for the determination of ethacrynic acid activity as described above. SDS²-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose and subsequent peroxidase staining of antibody-reactive proteins was carried out using a modified version of the method described by Towbin *et al.* (27) and Adams *et al.* (28). 4-Chloro-1-naphthol was used as peroxidase substrate. All the samples from any particular antibody were run on the same SDS gel so that a direct comparison could be made between them. Semiquantitation of the peroxidase staining bands was carried out by scanning using a Joyce-Loebel laser gel scanner. Values were obtained by scanning the entire band, and the resulting peak areas, which reflected band area and density, were integrated. Results were expressed as percentages of relevant controls calculated as the mean value \pm the standard deviation for three experiments.

RESULTS

The effect of long-term treatment of mice with interferon on the metabolism of various glutathione transferase substrates is shown in Table I. The metabolism of ethacrynic acid was significantly increased to 219% of control values by interferon treatment. The metabolism of 2,4-dichloro-1-nitrobenzene (DCNB) was significantly reduced (33%). In these experiments no change in the metabolism of the other substrates tested was measured. The Coomassie Blue staining pattern of mouse cytosolic fractions from control animals or animals treated with interferon is shown in Fig. 1. A signifi-

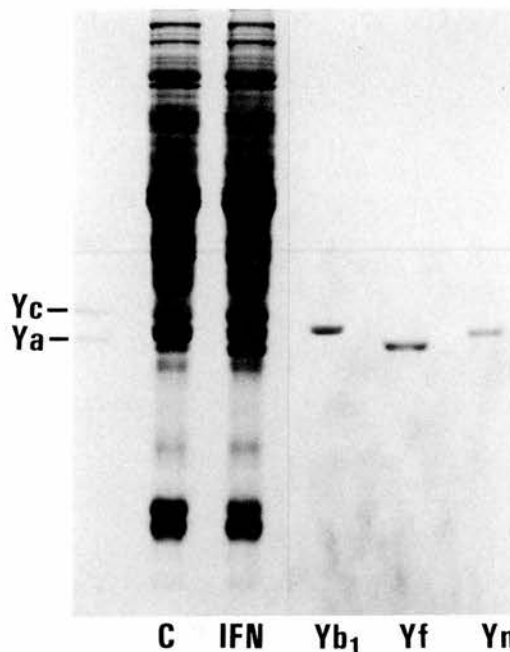


FIG. 1. Effect of interferon on the Coomassie Blue staining pattern of hepatic cytosolic proteins following SDS electrophoresis. Nude mice were treated with interferon (2×10^6 units/day for 30 days). Electrophoresis was carried out on 12% gels with 30 μg of cytosolic protein/track. The rat Ya, Yc, and Yb₁, and Yn subunits and the human Yf subunit were run as standards. C = control, IFN = interferon-treated samples.

cant induction of a low molecular weight band, M_r 24,800, and the suppression of bands in the region M_r 26,000–26,500 were observed. There also appeared to be some induction of a protein with an apparent M_r of 28,000. To assess the glutathione transferase subunits affected by interferon treatment, Western blots were probed with antibodies raised to the isolated rat and human enzymes. The reactivity of these antibodies with mouse cytosol and the molecular weights of the proteins identified are shown in Fig. 2 and Table II. All of the antibodies recognized structurally analogous proteins in the mouse. The apparent molecular weights of proteins identified were very similar to the rat or human counterparts with the exception of a mouse polypeptide which cross-reacted with antisera against the rat Yc subunit (M_r 27,500) but exhibited a faster mobility in the mouse with an apparent M_r of 25,000. The effect of interferon administration on the relative levels of the mouse proteins is shown in Figs. 3 and 4. The expression of some of the transferase isozymes was significantly suppressed by interferon administration. The degree of suppression appeared to vary considerably, even between isozymes, *i.e.* between Yb₁ and Yb₂, within the same gene subfamily. The Ya, Yb₁, Yb₂, Yc, Yk, B₁, and human Yb

¹ Glutathione transferase subunits Ya, Yc, Yb₁, and Yb₂ are those described as 1, 2, 3, and 4, respectively (4). The human transferases ϵ (B₁ B₁) and λ (Yf Yf) have been described as basic and acidic glutathione transferase, respectively (4). The acidic form has also been referred to as π and ρ .

² The abbreviation used is: SDS, sodium dodecyl sulfate.

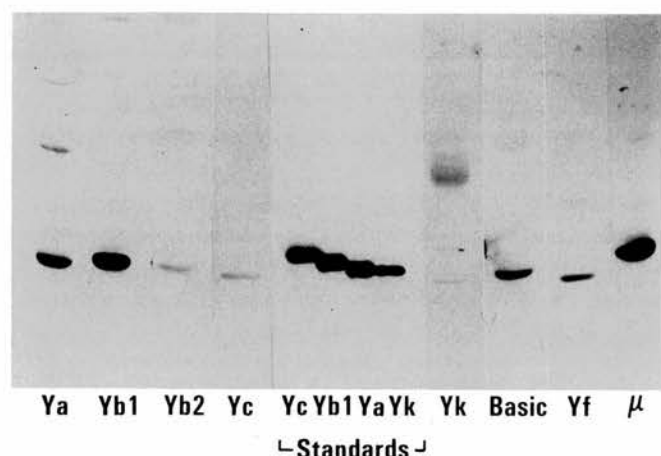


FIG. 2. Determination of mouse cytosolic glutathione transferases structurally related to those in rat and human. A single SDS gel was run with tracks containing either 30 μ g of cytosolic protein or pure rat transferases. Following transfer to nitrocellulose, the paper was cut into several strips and each one stained with a specific antibody. The molecular weights of the proteins identified were calculated from the mobilities of the staining bands relative to the mobilities of rat glutathione transferases used as standards.

TABLE II

Subunit molecular weights of mouse glutathione transferases identified with antibodies to the rat or human enzymes

Experimental details are given in the legend to Fig. 2. Weakly cross-reacting polypeptides are in parentheses.

Species	Glutathione transferase class	Glutathione transferase Subunit	Molecular weight in rat or in man	Molecular weight of cross-reacting protein in mouse
Rat	I	Ya	25,500	26,000
	I	Yc	27,500	(25,800)
	I	Yk	25,000	(25,800/27,500)
	II	Yb ₁	26,300	26,400
	II	Yb ₂	26,300	26,400
Man	Basic	B ₁	26,000	26,000
	Neutral	μ	26,700	26,400
	Acidic	Yf	24,800	24,800

polypeptides were suppressed 11, 11, 44, 30, 12, 47, and 14%, respectively. In contrast to these findings, a significant elevation in the level of the Yf subunit was measured. In three separate determinations this subunit was elevated to levels an average of five times higher than control animals (Figs. 3 and 4), a 7-fold increase being the maximum observed. In order to determine whether this change was observed on shorter interferon treatment, BALB/c mice were treated for 3 days with the same dose of interferon (Fig. 5). As with the nude mice, a significant elevation in the expression of the Yf subunit was measured in the interferon-treated animals. In order to establish that the elevation in the Yf subunit would account for the increase in ethacrynic acid activity, the enzyme was immunoprecipitated from the cytosol using the human antibody (Fig. 6). In agreement with previous findings (26), this enzyme is primarily responsible for ethacrynic acid activity in mouse cytosol. Indeed, all the activity could be immunoprecipitated using the human antibody to the acidic subunit.

DISCUSSION

The induction of mammalian glutathione transferases by exogenous agents is well documented (4). The results of this present study demonstrate that these enzymes are also regulated by endogenous agents such as interferons. Mouse inter-

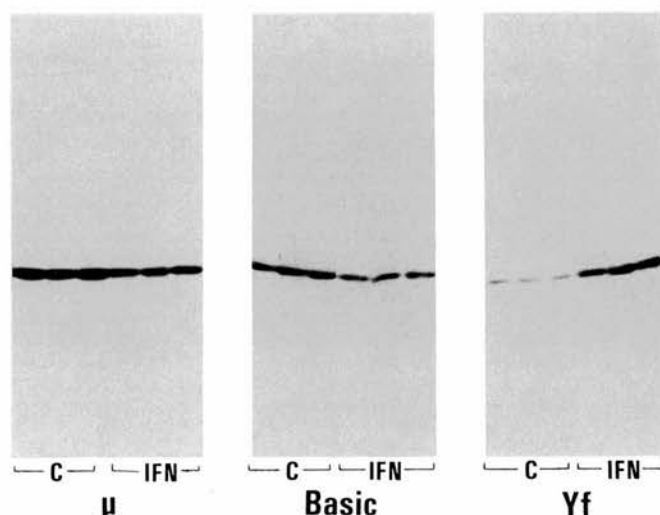


FIG. 3. Effect of interferon on the expression of individual glutathione transferase subunits in murine hepatic cytosol structurally related to the human isozymes μ (Yb Yb), λ (Yf Yf), and ϵ (Basic). Test animals received 2×10^5 units/day for 30 days. 12% polyacrylamide gels were used with 30 μ g of protein/track. Western blots were carried out using a modified method of that described by Towbin *et al.* (27) as described under "Materials and Methods." Three separate experiments are shown for each determination.

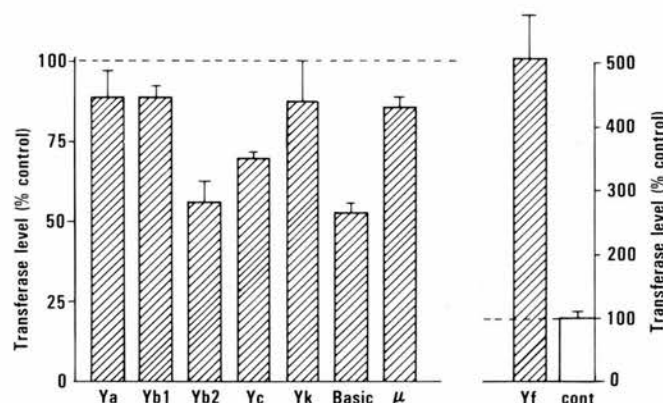


FIG. 4. Determination of relative changes in mouse hepatic glutathione transferase levels following interferon administration. Experimental details are given in Fig. 3. Western blots were photographed and then scanned with a Joyce-Loebel laser gel scanner. The entire band for each determination was scanned, and relative values were obtained by integration of the peak areas obtained. The values obtained are the means \pm S.D. from three separate experiments.

feron- α/β caused a significant suppression of the levels of certain glutathione transferases in the mouse liver. This was a differential effect where certain isozymes were only marginally suppressed, whereas others were reduced by up to 50%. Each glutathione transferase has a unique spectrum of enzymic activity (4), and these observations indicate that viral infection and other factors which induce interferon production will alter the detoxification of chemical toxins and carcinogens within the liver and possibly other tissues. The isozyme recognized by the antibody to transferase μ was suppressed to a similar extent to the Yb₁ subunit, and the cross-reactivity of this antibody with Yb₁³ suggests that two antibodies are recognizing the same protein. Antisera raised against the human basic glutathione transferase (B₁) and rat

³ P. K. Stockman and J. D. Hayes, unpublished.

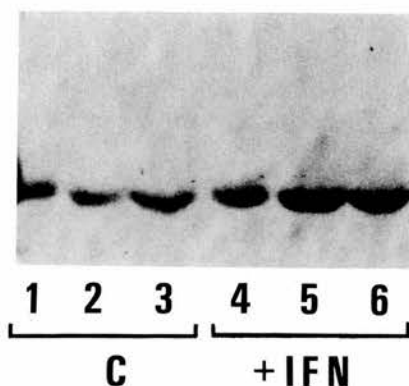


FIG. 5. Effect of interferon treatment on the expression of the glutathione transferase Yf subunit in female BALB/c mice treated for 3 days, 2×10^5 units/day. The Western blot was carried out on hepatic cytosol using the antibody to transferase λ (Yf Yf) as described under "Materials and Methods." Lanes 1-3 are cytosol from untreated animals. Lanes 4-6 are cytosol from animals treated with interferon.

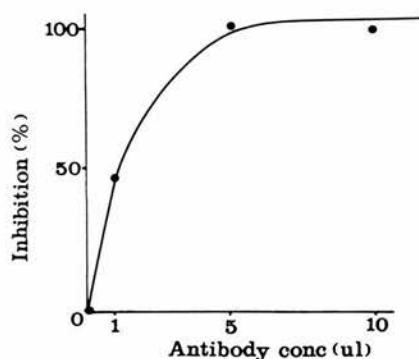


FIG. 6. Effect of immunoprecipitation of the Yf subunit on cytosolic ethacrynic acid activity. Mouse liver cytosol (100 μ l) from interferon-treated animals was incubated for 12 h at 4 $^{\circ}$ C with varying amounts of antibody to the human acidic (λ) subunit (1-15 μ l) and the volume made up with preimmune serum. After this period, 30 μ l of sheep anti-rabbit serum was added to 30 μ l of the sample and the mixture left for a further 3 h. The sample was then spun, and aliquots were taken for the determination of ethacrynic acid activity as described under "Materials and Methods." The control value was 146 nmol of conjugate formed/min/mg of protein.

Ya glutathione transferase subunits both cross-react with the mouse B₁ B₁, M₁, 26,000 glutathione transferase subunit, but the suppression observed with anti-basic was much more marked than that observed using the antibody to Ya; this probably reflects a closer immunochemical identity between the human and mouse glutathione transferase than between the corresponding rat and mouse enzymes. Slight variation between the molecular weights of the proteins identified was also measured, but these differences are within the limits of experimental error. In contrast to the suppression of some of the glutathione transferases, the marked induction of the Yf subunit (at least 5-fold) by interferon treatment was particularly striking. The changes in protein levels were to some degree qualitatively confirmed by changes in substrate metabolism. The metabolism of ethacrynic acid which was clearly mediated by the Yf subunit (Fig. 6) (26) was elevated, and the metabolism of DCNB, a substrate for the neutral (Yb) subunits, was reduced. The reason why DCNB activity did not change significantly could be explained by the balance between enzyme induction and suppression. The differential induction of mRNAs coding glutathione transferase has been reported by Pickett *et al.* (29), but there are few data in the literature describing suppression of glutathione transferase.

The Yf glutathione transferase subunit is only expressed at low levels in the livers of most species, including female mice, but is present in several extrahepatic tissues (8). It has been isolated and characterized from human placenta and lung (30). Very little is known about the regulation of the Yf subunit, and it appears to be refractory to induction by exogenous inducing agents. The induction of this subunit by interferon is therefore intriguing. The level of the Yf subunit is significantly increased in hepatic preneoplastic lesions, although other glutathione transferases are also elevated (33, 34). The increased levels of the transferases in these lesions will increase the resistance of these cells to cytotoxic insult. These changes are therefore thought to provide the preneoplastic cells a selective advantage important for neoplastic transformation (17, 30).

In various other examples of resistance to cytotoxic agents, glutathione transferases have been shown to be elevated. These include the resistance of normal bone marrow granulocytes to the cytotoxic effects of cyclophosphamide (35, 36) and the acquired resistance of tumor cells to cytotoxic drugs (18, 37, 38). An acidic transferase polypeptide, probably the Yf subunit, has recently been identified in an MCF 7 breast tumor cell line made resistant to adriamycin (18). We have also observed an elevation of this subunit in drug-resistant tumor cells.⁴ This isozyme therefore appears closely related to the resistance of both normal and neoplastic cells to cytotoxins.

The induction of the Yf subunit both by interferon and in response to chemical insult raises the intriguing possibility that there may be some common molecular changes in drug resistance and interferon-induced resistance to viral infection. Interferons selectively change molecular events in the cell. Although the overall rate of RNA and protein synthesis is unchanged, up to 100 mRNAs are induced and others are inhibited by interferon treatment (39, 40). To date, only a relatively few interferon-inducible gene products have been identified. These include two enzymes, a protein kinase and 2',5'-oligoadenylate synthetase as well as molecules of the major histocompatibility complex, metallothionein II, and thymosin (39, 40).

It is known that viral infection can give rise to very high localized tissue concentrations of interferon. As interferons have the capacity to alter both cellular glutathione transferase as well as cytochrome P-450 levels (15, 16), viral infection or inflammation may alter the response of cells to cytotoxins and chemical carcinogens. This could be either in the rate of activation of the compounds to ultimate carcinogens or in the rate of their detoxification. We are currently studying these possibilities.

The use of interferon in conjunction with cytotoxic drugs in cancer chemotherapy is being tested in clinical trials. The effects of interferon on drug-metabolizing enzymes may significantly change the metabolism and disposition of chemotherapeutic agents administered concomitantly. These data suggest that care must be taken when using such regimens to avoid side effects that may arise because of an altered capacity to detoxify and eliminate the cytotoxic drugs used.

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Reduced Levels of Drug-induced DNA Cross-Linking in Nitrogen Mustard-resistant Chinese Hamster Ovary Cells Expressing Elevated Glutathione S-Transferase Activity

Craig N. Robson, Alex D. Lewis, C. Roland Wolf, John D. Hayes, Andrew Hall, Stephen J. Proctor, Adrian L. Harris, and Ian D. Hickson¹

Departments of Clinical Oncology [C. N. R., A. L. H., I. D. H.] and Hematology [A. H., S. J. P.], University of Newcastle upon Tyne, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, and Imperial Cancer Research Fund Laboratory of Molecular Pharmacology and Drug Metabolism, Department of Biochemistry [A. D. L., C. R. W.], and Department of Clinical Chemistry, The Royal Infirmary [J. D. H.], University of Edinburgh, Edinburgh EH3 9YW, United Kingdom

ABSTRACT

We have reported previously (C. N. Robson *et al.*, *Cancer Res.*, 46: 6290-6294, 1986) the isolation of a Chinese hamster ovary cell line, designated CHO-Chl^r, that exhibits resistance to bifunctional nitrogen mustards while maintaining the normal parental level of sensitivity to several other alkylating agents. We have compared the rate of formation and repair of DNA cross-links induced by mechlorethamine in CHO-Chl^r and parental CHO-K1 cells, both in intact cells and in isolated nuclei. Equimolar doses of mechlorethamine induce significantly fewer DNA interstrand cross-links in CHO-Chl^r cells than in CHO-K1 cells, but levels of DNA-protein adducts are approximately equivalent in the two lines. There is a correlation between the relative resistance of CHO-Chl^r cells to mechlorethamine (34-fold) and the amount of drug required to induce approximately equal numbers of DNA interstrand cross-links in the two cell lines. This strongly implicates DNA-DNA adducts in the cytotoxic action of mechlorethamine. DNA cross-linking studies on isolated nuclei reveal only minor differences between the two lines even with identical drug treatments. The rate of cross-link repair is comparable in the two cell lines. These results, taken together with our earlier observation that the rate of drug accumulation is identical in these two lines, suggest that enhanced cytoplasmic drug detoxification is the underlying resistance mechanism in CHO-Chl^r cells. We have measured cellular glutathione S-transferase activity, using both the general substrate 1-chloro-2,4-dinitrobenzene, and substrates with some specificity for the different classes of transferase isoenzymes. Total enzyme activity (as measured with 1-chloro-2,4-dinitrobenzene) is elevated 3-fold in the resistant cells. A 2- and 5-fold increase, respectively, in activity against ethacrynic acid and cumene hydroperoxide is detectable in CHO-Chl^r cells. This elevation in catalytic activity in the resistant cells is reflected in higher levels of both the Y_r- and Y_s-type transferase subunits.

INTRODUCTION

Alkylating agents represent an important class of antitumor drugs. However, as with several other groups of clinically useful cytotoxic agents, their effectiveness is limited by the emergence of subpopulations of resistant tumor cells. Several mechanisms of resistance to alkylating agents in human and rodent cell lines have been reported, including reduced membrane permeability, increased efficiency of DNA repair, and enhanced drug detoxification (1-10). Indeed, the mechanism responsible for nitrogen mustard resistance is frequently multifactorial. For example, a melphalan-resistant CHO² cell line was reported to have an increased level of sulfhydryl groups, enhanced drug efflux, and a decrease in drug-induced DNA cross-linking (1).

Several alkylating agents are sufficiently electrophilic to react directly with the major cellular non-protein thiol-containing

compound, GSH. However, mammalian cells have evolved a supergene family of enzymes, the GSTs, which catalyze this conjugation reaction (11). The GSTs are believed to be responsible for catalyzing the conjugation of GSH to electrophilic metabolites formed from nitrogen mustards (12, 13). The resultant decrease in the steady state level of reactive drug metabolites spares critical intracellular targets such as DNA from levels of alkylation that cannot be repaired effectively.

We reported recently the isolation of a CHO cell line (CHO-Chl^r) with acquired resistance to bifunctional nitrogen mustards (14). This subline was isolated on the basis of chronic exposure to chlorambucil and exhibits resistance not only to chlorambucil (24-fold) but also to melphalan (14-fold) and mechlorethamine (34-fold). In contrast to the chlorambucil-resistant Walker rat carcinoma (WR) cell line (15), CHO-Chl^r cells are not cross-resistant to other bifunctional alkylating agents, such as mitomycin C, *cis*-diamminedichloroplatinum(II) or cyclophosphamide, nor are they collaterally sensitive to nitrosoureas.

We have investigated the mechanism of resistance to nitrogen mustards in CHO-Chl^r cells. Evidence is presented that the principal alteration found in the resistant cells following exposure to drug is a reduction in the extent of damage to DNA. This reduction is accompanied by an apparent increased rate of drug detoxification that is a consequence of an elevation in GST activity coupled with a small increase in cellular GSH content.

MATERIALS AND METHODS

Cell Culture and Media. Cells were routinely maintained in Ham's F-10 medium supplemented with 5% fetal calf serum, 5% newborn calf serum, glutamine (3 mM), and antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml; and nystatin, 50 units/ml). Cells were grown as monolayers at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Labeling and Alkaline Elution. Cellular DNA was labeled by incubating cells with [2-¹⁴C]thymidine (0.02 µCi/ml) for 48 h. Following growth in isotope-free medium for 16 h, cells were exposed to mechlorethamine for 1 h and, where indicated, then incubated in drug-free medium to follow the time course of DNA repair.

DNA-protein and DNA-DNA interstrand cross-links were assayed by the method of alkaline elution, essentially as described by Kohn *et al.* (16). Briefly, approximately 5 × 10⁵ cells (which were X-irradiated on ice prior to elution) were impinged onto polyvinyl chloride filters (2-µm pore), lysed with 40 mM EDTA-2 M NaCl-0.2% sodium dodecyl sarkosine (pH 10.0), and, where indicated, exposed to 0.5 mg/ml proteinase K for 1 h. The DNA was eluted from the filters at a rate of 0.035 ml/min using a solution of 20 mM EDTA (free acid) adjusted to pH 12.1 with tetrapropylammonium hydroxide (for the measurement of total cross-links). For DNA-DNA cross-links, the elution buffer contained 0.1% sodium dodecyl sulfate. Fractions were collected at 90-min intervals over 15 h.

The use of a Watson-Marlow 202U/AA 16 channel constant speed peristaltic pump gave highly reproducible flow rates in all channels.

Nuclei were prepared by the method of Glisson *et al.* (17). Isolated

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¹ To whom requests for reprints should be addressed.

² The abbreviations used are: CHO, Chinese hamster ovary; GST, glutathione S-transferase; GSH, glutathione; PBS, phosphate-buffered saline; TBST, 50 mM Tris-HCl (pH 7.9)-0.15 M NaCl-0.05% (v/v) Tween 20.

nuclei were exposed to drug in PBS at 37°C and then harvested and resuspended in ice-cold PBS prior to X-irradiation (600 rads).

Cross-linking factors are defined as the ratio of the log fraction DNA retained after 9 h in the irradiated control and the log fraction DNA retained in the sample treated with both mechlorethamine and radiation, as described by Ross *et al.* (18).

Glutathione S-Transferase Assays. GST activity towards the substrates 1-chloro-2,4-dinitrobenzene and ethacrynic acid was measured by the method of Habig and Jakoby (19). Peroxidase activity towards cumene hydroperoxide was carried out as described by Stockman *et al.* (20). Cell suspensions were sonicated and spun for 5 min at 13,000 rpm in an Eppendorf microfuge, and the supernatants were assayed.

Dot Blots. This was performed to measure the relative levels of the different classes of GSTs in the 2 cell lines. The antisera used were raised in rabbits against the human proteins GST λ , GST μ , and GST B_1B_1 , which are members of the families referred to as "acidic," "neutral," and "basic" class GSTs, respectively. The specificities of these antisera towards purified rat, mouse, guinea pig, and hamster GSTs have been described elsewhere (21, 22).

Cytosolic proteins from CHO-Chl' and CHO-K1 cells were dotted onto nitrocellulose filters, washed for two 10-min periods in TBST, and blocked for 1 h with 3% (w/v) bovine serum albumin in TBST. Two further 10-min washes with TBST were carried out, after which filters were incubated for 1 h with a specific human antibody to each of the three GST isoenzyme classes. Following four 15-min washes with TBST, the filters were incubated for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase antibody. The filters were washed and dots were visualized either using 4-chloro-1-naphthol or by autoradiography after labeling with 0.19 MBq 125 I-protein A.

Immunocytochemistry. Immunocytochemical staining of air-dried cytocentrifuge preparations of intact cells was performed using an adaptation of the method of Swirsky *et al.* (23). Briefly, cells were fixed for 2 min in methanol:acetone (1:1, v/v) and then washed with PBS. A 100- μ l aliquot of primary antibody (raised against human acidic, neutral, or basic forms of GST) was added to each slide and incubated at room temperature for 45 min. Control slides were incubated with Earle's balanced salt solution. Following incubation, slides were washed with PBS and immersed for 10 min in 100 ml of methanol:7 ml of 30% (v/v) hydrogen peroxide. After further washing with PBS, 100 μ l of secondary antibody (swine anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:40 in Earle's balanced salt solution containing 5% normal inactivated human serum) were added to each slide and incubated for 45 min. Further washing with PBS was followed by development of peroxidase for 10 min in a mixture of 50 mg of 3,3'-diaminobenzidine in 100 ml of PBS and 20 μ l of H_2O_2 . Slides were then washed in distilled water and counterstained with hematoxylin.

RESULTS

DNA Cross-Linking in Resistant and Parental CHO Cells. DNA cross-linking induced by mechlorethamine was measured in CHO-Chl' and CHO-K1 cells using alkaline elution (16). Representative elution profiles for CHO-K1 cells following a 1-h exposure to different concentrations of drug are shown in Fig. 1. The effect of increasing doses of mechlorethamine is to reduce the rate of DNA elution, reflecting the formation of DNA cross-links.

The level of both DNA interstrand and DNA-protein cross-links was measured in resistant and parental cells. DNA interstrand cross-linking frequencies were determined by including proteinase K in the elution protocol, in order to eliminate DNA-protein adducts. The level of DNA interstrand cross-linking induced by equimolar doses of mechlorethamine is very significantly higher ($P < 0.001$) in CHO-K1 cells than in CHO-Chl' cells (Fig. 2). CHO-Chl' cells have previously been shown in clonogenic survival assays to be 34-fold resistant to mechlorethamine (14). Based on this degree of resistance, it can be seen (Fig. 2) that approximately equitoxic doses of drug induce the

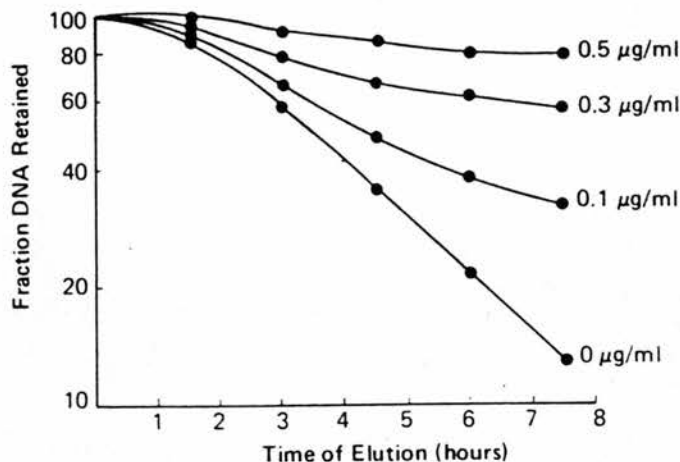


Fig. 1. Representative filter elution profiles for CHO-K1 cells following a 1-h exposure to different concentrations (as indicated) of mechlorethamine. In all cases, cells were X-irradiated with 600 rads immediately prior to cell lysis and elution.

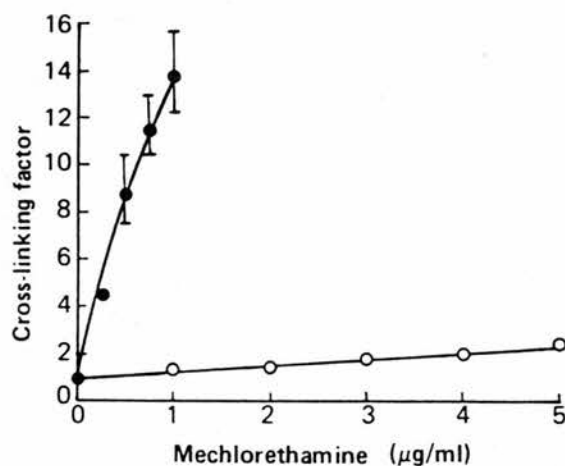


Fig. 2. Levels of DNA interstrand cross-links induced in CHO-K1 (●) and CHO-Chl' (○) cells, as a function of mechlorethamine concentration. Cross-linking factors were determined as described in "Materials and Methods." Points, means of at least 3 independent experiments; bars, SE.

same level of DNA-DNA adducts in the 2 cell lines. For example, mechlorethamine doses of 0.12 and 4 μ g/ml, respectively, produce a cross-linking factor of 2 in parental and resistant cells.

Levels of DNA-protein cross-links were assessed by calculating the difference between total cross-links (absence of proteinase K) and DNA interstrand cross-links. Both lines accumulate similar numbers of DNA-protein cross-links, although the resistant cells show a small but consistently increased level of these adducts (data not shown). Thus, the strikingly higher level of DNA cross-links seen in CHO-K1 cells appears to be solely a consequence of an increase in the number of DNA-DNA adducts.

Repair of DNA Cross-Links. To assess the DNA repair proficiency of the two lines, cells were exposed for 1 h to a dose of mechlorethamine that induced the same level of DNA interstrand cross-links, before being washed and returned to drug-free medium. At intervals, samples were taken and the degree of cross-link repair was determined. Fig. 3 shows that the rate of cross-link repair is not significantly different in parental and resistant cells.

Cross-Linking in Isolated Nuclei. To determine whether the low level of drug-induced DNA damage in CHO-Chl' cells is a consequence of a reduced level of active drug being accumulated

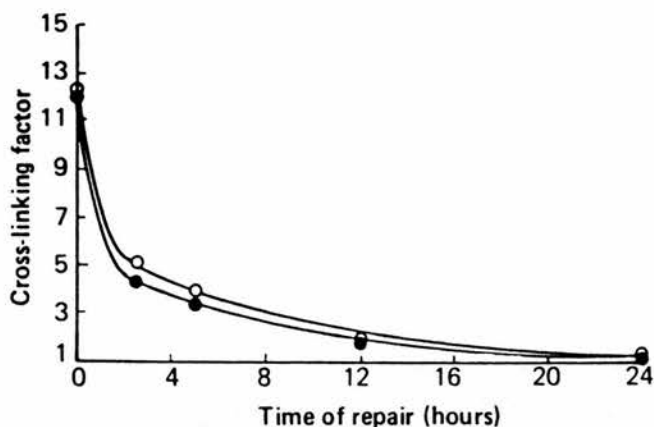


Fig. 3. Kinetics of repair of DNA interstrand cross-links induced by mechlorethamine in CHO-K1 (●) and CHO-Chl' (○) cells. Points, means of 2 independent determinations.

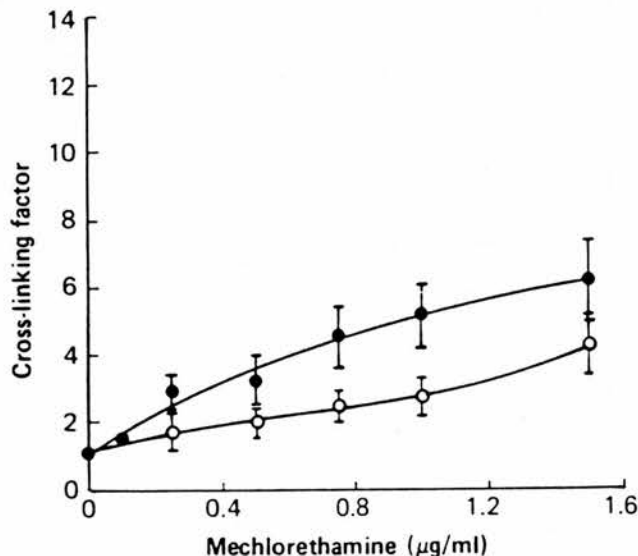


Fig. 4. Levels of DNA interstrand cross-links induced in nuclei isolated from either CHO-K1 cells (●) or CHO-Chl' cells (○), as a function of mechlorethamine concentration. Points, means of at least 3 independent experiments; bars, SE.

in the nucleus of whole cells, cross-linking measurements were repeated using isolated nuclei. In this case, far lower concentrations of mechlorethamine were required to give measurable levels of cross-links. Although slightly higher numbers of cross-links were induced in nuclei isolated from parental CHO-K1 cells than those from the resistant cells (Fig. 4), the difference is not significant ($P > 0.05$) and far less striking than that seen in similar studies with intact cells.

These results suggest either that lower levels of drug are accumulated intracellularly by CHO-Chl' cells or that drug detoxification in the cell cytoplasm occurs more efficiently. The former possibility can be discounted inasmuch as we have previously shown that the rate of drug accumulation is not significantly different in CHO-Chl' cells (14). Thus, drug detoxification would appear to be the most likely mechanism of resistance.

The GST family of isoenzymes has been reported to be involved in the detoxification of a variety of xenobiotics, including alkylating agents, via conjugation with reduced GSH (11, 12). The levels of these enzymes in the two cell lines were therefore measured.

Measurements of GST Activity. Initially, transferase activity using 1-chloro-2,4-dinitrobenzene as substrate was measured in parental and resistant cells. Activity against 1-chloro-2,4-dini-

trobenzene reflects overall levels of the different transferase isoenzymes. Fig. 5 shows that CHO-Chl' cells have a 3-fold higher total GST activity. In order to obtain an indication of the enzyme subgroups that are overexpressed, assays were performed using substrates that show a degree of specificity for the neutral (μ , Y_bY_b), acidic (λ , Y_fY_f), or basic (ϵ , B_1B_1) isoenzyme forms. No activity against styrene oxide (for the neutral isoenzymes) was detected. However, a significant elevation in both cumene hydroperoxide (5-fold) and ethacrynic acid (2-fold) activities was seen. These activities are indicative of the basic and acidic GST isoenzymes, respectively, although more than 1 class of GST may show significant activity with these substrates.

Immunological Measurements of GST Levels. To confirm that the increased catalytic activity against ethacrynic acid and cumene hydroperoxide was reflected in increased GST levels, immunological measurements of GST levels were performed. Higher levels of cross-reaction with the Y_f -specific antisera (acidic) and, more particularly, the Y_b -specific antisera (basic)

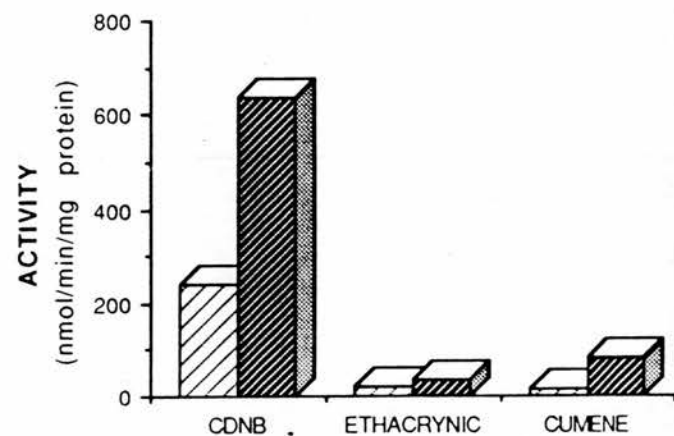


Fig. 5. Histogram of 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, and cumene hydroperoxide activities in CHO-K1 (□) and CHO-Chl' (■).

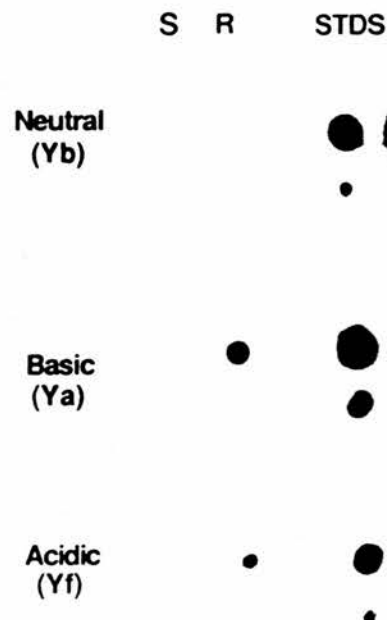


Fig. 6. Autoradiogram of "dot blots" of cytosolic proteins from CHO-K1 (S) and CHO-Chl' (R) cells. Either 2.5 μg (upper dot) or 0.25 μg of cellular protein was dotted onto nitrocellulose filters, which were processed as described in "Materials and Methods." The column labeled STDS represents GST standards (0.25 and 0.025 μg in each case) of the different classes of GST.

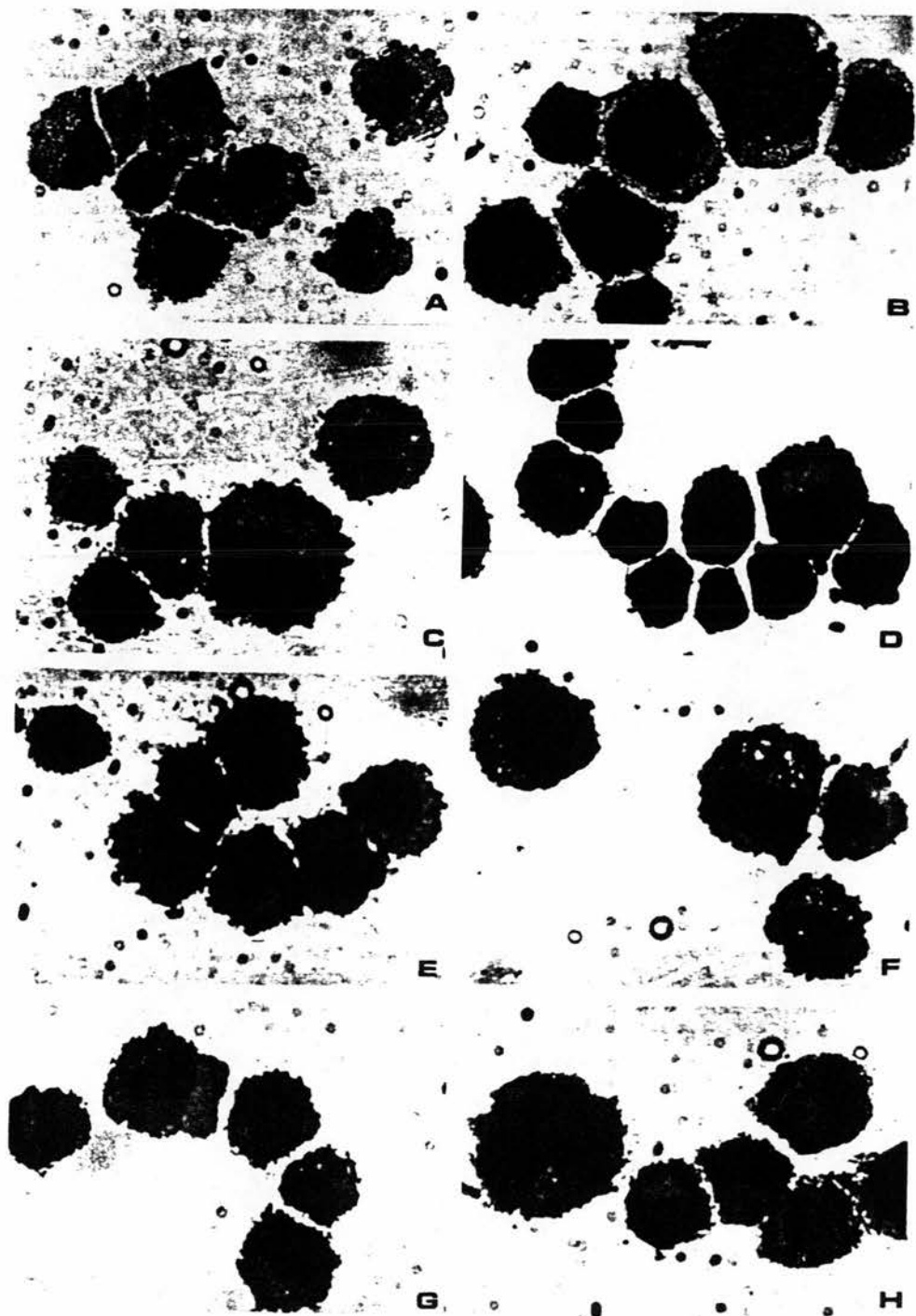


Fig. 7. Immunocytochemical analysis of fixed CHO-K1 (A, C, E, and G) and CHO-Chl' (B, D, F, and H) cells. Cells were exposed to either no antibody (A and B) or antibodies specific for the human basic (C and D), neutral (E and F), or acidic (G and H) isoenzyme forms. Treatment conditions are outlined in "Materials and Methods." The intensity of staining is an indication of the intracellular GST concentration.

are seen in CHO-Chl' cells, as revealed by either dot blotting (Fig. 6) or immunocytochemical analysis (Fig. 7).

DISCUSSION

We have studied the level of DNA damage induced by mechlorethamine in cell lines resistant or sensitive to bifunctional nitrogen mustards. The striking difference between cross-link levels seen in intact cells, but not in isolated nuclei, is indicative of reduced intranuclear accumulation of active drug in the resistant cells. Because total drug uptake into whole cells is apparently unchanged in CHO-Chl' cells (14) it appears likely that an increase in cytoplasmic drug detoxification is the underlying mechanism of drug resistance in these cells.

Intracellular glutathione has a major role in the detoxification

of reactive metabolic intermediates. Although spontaneous reactions with electrophilic molecules can occur readily *in vitro*, mammalian cells contain a variety of GST isoenzymes that appear to catalyze these reactions *in vivo*. A large number of distinct GST isoenzymes have been purified from human, mouse, and rat cells, and in each species the different forms have been shown to be members of three separate families. In humans, mice and rats, GSTs that are members of the same family display immunochemical cross-reactivity, exhibit similar catalytic properties, and possess sequence homology (11, 24). The GSTs in the Chinese hamster have not been well characterized (25, 26), but our data indicate that the three families are represented in this species.

Changes in the intracellular level of GST activity have been reported previously in alkylating agent-resistant cell lines. For

example, the chlorambucil-resistant Walker carcinoma WR cell line has 2-fold higher GST activity than the parental WS line, although in 2 cases (ethacrynic acid and *trans*-4-phenyl-3-buten-2-one), subunit specific activities are higher in the parental cells (7). In a Yoshida cell line resistant to cyclophosphamide, an approximately 6-fold higher GST activity was seen compared to the sensitive parental line (4). An *in vivo* model for drug resistance in ovarian cancer also implicates elevated GSH and GST levels (27). In our CHO-Chl' cells, GST activity is elevated 3-fold, which appears to be a consequence of an increase in the level of 2 enzyme forms. Whether the M_r 25,000 protein previously reported to be overproduced in CHO-Chl' cells (14) is a GST subunit and associated with the observed increase in GST activity in these cells is currently under investigation. Although CHO-Chl' and the Walker WR cells show comparable levels of resistance to nitrogen mustards (14, 15), CHO-Chl' cells differ from WR cells in being sensitive to other classes of bifunctional alkylators, such as *cis*-diamminedichloroplatinum(II) and mitomycin C. The narrow range of drugs to which CHO-Chl' are resistant indicates that this line may represent a good model for studying the substrate specificities of the different transferase isoenzymes and their cellular function. This may be particularly relevant because of the apparent lack of accompanying changes in the resistant cells, such as membrane permeability.

Analysis of DNA damage showed a dramatic reduction in the level of DNA cross-linking induced by mechlorethamine in CHO-Chl' cells. This difference was almost entirely due to the formation of DNA-DNA cross-links, not DNA-protein adducts. Indeed, it appears that slightly more DNA-protein cross-links were seen in the resistant than the parental cells at equimolar drug doses. If the GSTs were inactivating nitrogen mustard-derived nucleophiles, it may be expected that monoadducts as well as cross-links would be prevented or quenched, and hence the level of DNA protein cross-links would also be reduced in CHO-Chl' cells. It is possible that GSTs are able to inactivate monoadducts already bound to DNA by conjugation with GSH or that the GSH-conjugated nitrogen mustard derivatives are still capable of monovalent attack and that this could produce equivalent levels of DNA protein cross-links in the two lines. Further experiments are required to address these possibilities. There is evidence that GSH-conjugated nitroimidazole derivatives can still radiosensitize (28) and certain GSTs are bound to interchromatic regions of the cell nucleus (nonhistone protein BA is a Yb₁Yb₁ glutathione transferase) (29).

Our results support the contention that the principal lethal lesion generated by nitrogen mustards is the DNA interstrand cross-link. There appears to be no correlation between the formation of DNA-protein adducts and the cytotoxic effects of mechlorethamine. The apparent relationship between increased drug detoxification and a reduction in the level of DNA cross-linking provides additional evidence that GST can directly protect cells against the lethal effects of alkylating agents. The minor difference in cross-link levels seen in experiments with isolated nuclei may reflect the presence of low levels of GST in the nuclei of normal cells or of contamination by cytoplasmic components of the prepared nuclei.

Our previous observation that the steady state intracellular GSH content of CHO-Chl' cells is elevated approximately 2-fold (14) indicates that acquired drug resistance in these cells is presumably mediated both by this increase in acceptor sulfhydryl concentration and the overexpression of specific GST subunits.

The rate of repair of DNA-DNA adducts is apparently un-

changed in the resistant cells. In contrast to that seen previously in a melphalan-resistant CHO cell line (1), the rate of repair of mechlorethamine-induced cross-links is relatively rapid in our cells and cross-link numbers did not reach a peak 3 h after the removal of drug. It is difficult to make definitive statements concerning the similarity in DNA repair capacity of 2 cell lines, particularly when the levels of cross-links measured represent a balance between continuing formation and loss by repair. However, it would appear that there are no gross changes in DNA repair capacity in CHO-Chl' cells.

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The role of glutathione in determining the response of normal and tumour cells to anticancer drugs

C. R. WOLF,* A. D. LEWIS,* J. CARMICHAEL,*†
D. J. ADAMS,*‡ S. G. ALLAN*§ and D. J. ANSELL*||

*Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, University Department of Biochemistry, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K., §I.C.R.F. Medical Oncology Unit, Edinburgh EH4 2XU, U.K., and †Department of Zoology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JT, U.K.

The treatment of cancer with anticancer drugs has progressed very slowly over recent years with only a few tumour types that can be cured with drugs alone. Some tumours are intrinsically resistant to drug therapy, whereas others which are initially sensitive to their effects after a period of time become resistant to the drugs used. This acquired drug resistance is one of the major reasons why ovarian, breast and small cell lung tumours cannot be cured. There are many mechanisms providing an explanation of the acquisition of drug resistance, including changes in tumour cell population, altered tumour cell growth rate of oxygenation. In addition, phenotypic changes may occur within the tumour cells as a direct result of drug treatment, which make them resistant. It is the latter form of acquired resistance which forms the basis of the studies outlined below. The effectiveness of an anti-cancer drug is dependent on the toxicity of the drug to the tumour relative to the normal cells of the body. The cells of the bone marrow are the major susceptible normal tissue. Most other tissues are also affected by one drug or other; however, the rapidly dividing cells of the gastrointestinal tract and the hair follicles are also prime targets.

Glutathione (GSH) and GSH-dependent enzymes, such as the glutathione transferases and glutathione peroxidase, provide a primary defence mechanism in cells against the lethal effects of toxic chemicals (Meister & Anderson, 1983).

Abbreviations used: GSH, glutathione; GSSG, oxidized glutathione; BSO, buthionine sulfoximine; MESNA, mercaptoethane sulphonate.

†Present address: Department of Radiotherapy, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 4LP, U.K.

‡Present address: Department of Microbiology, University of Leeds, Leeds LS2 9JT, U.K.

We therefore have been studying the regulation of these enzymes in both normal and tumour cells after their exposure to anticancer drugs. The anticancer drugs where GSH may play an important role in detoxification can be split into two major categories: drugs which are detoxified by direct conjugation with GSH and drugs where formation of reactive oxygen intermediates, such as superoxide and hydroxyl radicals, are involved. In the latter case, GSH detoxifies these reactive intermediates through oxidation to GSSG catalysed by glutathione peroxidases. To date there have been very few studies on the role of glutathione in the conjugation of anticancer drugs; however, there are some reports which indicate its potential importance (Hirono, 1960; Calcutt & Connors, 1963; Connors, 1966; Arrick & Nathan, 1984; Green *et al.*, 1984; Adams *et al.*, 1985; Hamilton *et al.*, 1985; Carmichael *et al.*, 1986a; Wolf *et al.*, 1986) (Table 1). The involvement of GSH in the dismutation of peroxides is unquestioned. It is also clear that several anticancer drugs will cause the formation of reactive oxygen intermediates. Whether cellular GSH concentrations are critical to the susceptibility of the cells to these compounds is still not clear. There are, however, some reports which suggest that this is the case (Doroshov *et al.*, 1980).

The relative concentration of GSH in normal and tumour cells will therefore be an important factor in determining therapeutic index. In addition, any changes in cellular GSH which occur within normal and/or tumour cells after drug treatment which make them resistant to the further drug administration, could also be a critical factor in therapy with chemotherapeutic agents. In order to study GSH regulation in normal cells after cytotoxic insult we have adopted a model where the prior administration of a low dose of cyclophosphamide to mice can protect against a normally lethal dose of the same compound, and in some cases other compounds (Millar *et al.*, 1975). In this case the protection is associated with protection of the bone marrow. This property has been termed 'drug priming'. In view of the potential involvement of GSH in detoxification of cyclophosphamide (Berrigan *et al.*, 1982) we studied whether the resistance of the bone marrow to high concentrations of cyclophosphamide after 'priming' might be related to changes in GSH homeostasis. On days 5, 6 and 7 after a 'priming'

Table 1. Role of GSH in the activity of anticancer drugs

Group	Compound	Interaction with GSH	Consequence
Alkylating agents	Nitrogen mustard	Conjugation	Detoxification
	Cyclophosphamide	Conjugation	Detoxification
	Melphalan	Conjugation	Detoxification
	Chlorambucil	Conjugation	Detoxification
	1,3-Bis(chloroethyl)-1-nitrosourea	Inhibition of glutathione reductase	(Activation)
Anti-metabolites	6-Thiopurine	Conjugation	Detoxification
	Methotrexate	Accelerated uptake	Activation
	Azathioprine	Conjugation	Activation
Other	Mitomycin C	Conjugation	Detoxification
	Adriamycin	Reduction of peroxides	Detoxification
	Mitoxantrone	Conjugation	Detoxification
	Bleomycin	Reduction of Fe(III)-bleomycin complex	Activation
	Neocarzinostatin	DNA cleavage requires GSH	(Activation)
	cis-Platinum	Chelation	(Detoxification)
	VP-16	(Conjugation)	(Detoxification)

dose of cyclophosphamide (75 mg/kg intraperitoneally) male CBA mice become almost completely resistant to a lethal dose of this compound (Kimball *et al.*, 1976). Bone marrow GSH levels were initially markedly suppressed; however, on days 5, 6 and 7, glutathione levels were increased 2–3-fold above control values. As this mirrored the days where maximum protection against a lethal dose was observed, the changes in GSH homeostasis could therefore represent a mechanism for the protection of the bone marrow. In substantiation of this possibility when mice were treated with buthionine sulfoximine (BSO) (a compound which depletes cellular GSH levels), the protection afforded by drug priming was partially reversed (Carmichael *et al.*, 1986b). Although this evidence is not conclusive, it demonstrates that GSH plays a role in the protection of cells against cyclophosphamide metabolites and also that the elevation in GSH in the bone marrow may be part of the protection mechanism. In addition to these conclusions it is perhaps more important that cells when exposed to a cytotoxin over-produce glutathione. As the glutathione transferase enzymes were also elevated at the same time points, this indicates that the changes observed may represent a general stress response (see also below). In order to determine which subpopulation of the bone marrow was involved in this response, different bone marrow cell populations were sorted using a fluorescence-activated cell sorter after priming. The erythrocyte and lymphocyte GSH levels were unchanged; however, a 2-fold elevation in granulocyte GSH and glutathione transferase activity was measured (Carmichael *et al.*, 1986a). This finding was in agreement with the observation that the lethal effects of cyclophosphamide are related to granulocytopenia. In addition to changes in bone marrow granulocytes, GSH and GST activity were also elevated in granulocytes isolated from the peripheral circulation (Fig. 1).

The overproduction of GSH in cells has been observed in various other models involving resistance of cells to cytotoxins, for example, in preneoplastic lesions in the liver (Demle & Oesterle, 1980) and in lungs of rats made tolerant to a lethal dose of oxygen (Kimball *et al.*, 1976). As suggested above the overproduction of GSH may therefore be a general response of cells to stress. Enzymes involved in GSH homeostasis have also been shown to be part of the

stress response in bacteria (Christman *et al.*, 1985). We are currently evaluating the role of the glutathione transferases as stress response proteins.

GSH levels in tumour cells will be a factor in their susceptibility to anticancer drugs. In this regard, we have been studying both the regulation of GSH and GSH-dependent enzymes in drug-sensitive and drug-resistant tumour cell lines in culture. Two ovarian cell lines, PE01 and PE04, have been derived from a patient before and after she becomes resistant to cis-platinum (Wolf *et al.*, 1987). Karyotyping showed the lines to be of the same clonal origin but to have diverged from each other. The cell lines showed differences in their sensitivity to cis-platinum and the alkylating agent chlorambucil. GSH levels were approx. 2-fold higher in the drug-resistant line. In addition, a variety of GSH-dependent enzymes were also changed. Glutathione transferase activity was elevated 2-fold and γ -glutamyltranspeptidase 10-fold. Similar to the normal and preneoplastic cell models, it would appear that a cluster of genes relating to glutathione metabolism may be important in making the cells drug resistant. Indeed there are now a variety of reports in the literature showing an elevation in GSH and glutathione transferase levels in tumour cell lines made resistant to anticancer drugs *in vitro* (Hirono, 1960; Calcutt & Connors, 1963; Batist *et al.*, 1985; Wang & Tew, 1985; Wolf *et al.*, 1985; McGowan & Fox, 1986).

In view of the central role of GSH in detoxification there is obvious interest in manipulating GSH levels in cells in a manner which will alter the normal to tumour cell GSH ratio and therefore sensitize the tumour cells to anticancer drugs. In order to do this one must first understand how GSH is regulated in cells. In this regard we have been studying levels of GSH in ovarian and other cell lines during the cell cycle. In both synchronized cells in S-phase or cells

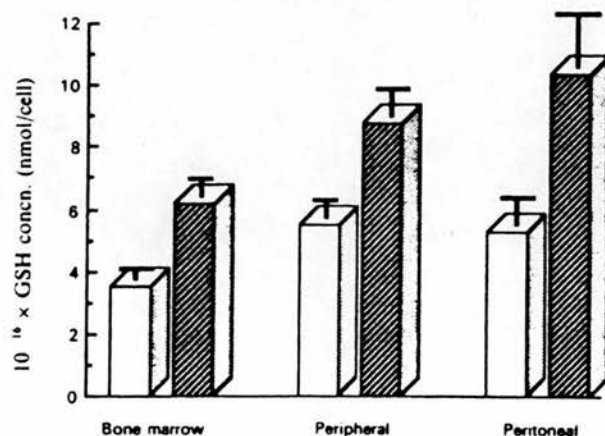


Fig. 1. Comparison of granulocyte GSH levels in untreated mice (□) or mice treated with cyclophosphamide (■).

Marrow and circulating granulocytes were obtained by fluorescence-activated cell sorting. Peritoneal granulocytes were induced by the injection of casein (2%) 4 h before withdrawal of the cells. Values were obtained 5 days after cyclophosphamide administration (75 mg/kg).

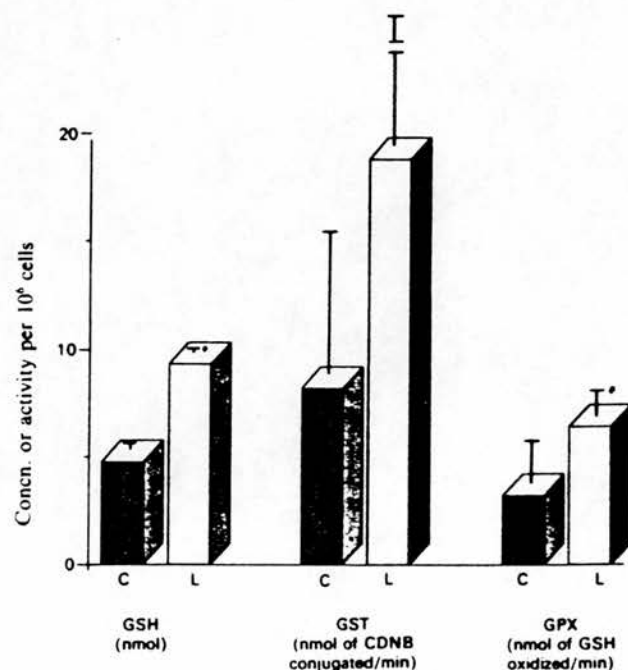


Fig. 2. GSH, glutathione peroxidase (GPX) and glutathione transferase (GST) in confluent cells (C) compared with cells in logarithmic growth (L).

GSH was determined by h.p.l.c. after monobromobimane derivatization, glutathione peroxidase was measured fluorimetrically using H_2O_2 as substrate and glutathione transferase measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. *Significantly different from controls: $P = < 0.05$.

in logarithmic growth, significantly higher levels of GSH are measured relative to non-dividing cells. In addition, the enzyme glutathione peroxidase is also elevated in dividing cells (Fig. 2). At the present time data for the glutathione transferases is inconclusive, although activities were also found to be elevated in dividing cells. Attempts to modulate cellular GSH levels have centred around the glutathione synthetase inhibitor BSO. As described above, administration of this compound will sensitize cells to the effects of cyclophosphamide. This compound has also been shown to sensitize tumour cells to anticancer drugs (Green *et al.*, 1984; Somfai-Relle *et al.*, 1984; Hamilton *et al.*, 1985; Crook *et al.*, 1986). Hamilton *et al.* (1985) have demonstrated that BSO will sensitize ovarian cell lines to the drug melphalan. The dependence of all cells on GSH as a protection mechanism against chemical or oxygen-radical damage, indicates that care should be taken in the use of potent GSH synthesis inhibitors such as BSO in cancer therapy. There has recently been a report which indicates that administration of BSO to mice increases the incidence of cataracts (Calvin *et al.*, 1986).

As an alternative to the depletion of GSH in the tumour as a method of sensitization towards anticancer drugs, another approach would be to reduce toxicity to normal tissues by administering compounds which either selectively elevate GSH levels in normal cells (Anderson *et al.*, 1985) or otherwise protect them from the drugs being used (Connors, 1966). Some compounds which contain sulphhydryl groupings are currently being used clinically in this manner. For example, the compound mercaptoethane sulphonate (MESNA) is used to protect the bladder against the toxic side-effects of cyclophosphamide and ifosfamide (Brack *et al.*, 1982). The compound *N*-acetylcysteine has also been shown to be effective in this regard (Berrigan *et al.*, 1982). MESNA is a drug which is selectively taken up into cells of the gastrointestinal tract and kidney (Ormsdahl *et al.*, 1983). The gastrointestinal tract and the kidney are two major targets for the toxic effects of cis-platinum, which is an antitumour drug which is poorly tolerated by patients due to the nausea it induces. In recent work we have shown that MESNA dosed orally or intraperitoneally into mice can significantly reduce the toxicity of cis-platinum to the gastrointestinal tract (Allan *et al.*, 1986). In addition, MESNA administration also significantly reduced cis-platinum lethality. A problem associated with the use of compounds which reduce toxicity to normal tissues is the possibility of reduced antitumour effect. When MESNA was dosed to mice already administered the leukaemia cell line L1210, the effectiveness of cis-platinum to increase survival time was not compromised (Calvin *et al.*, 1986). This indicates that it is possible to reduce the toxicity to normal tissues without reducing the therapeutic efficacy.

In conclusion, the regulation of GSH and GSH-dependent enzymes in normal and tumour cells is a potentially important determinant in therapeutic response in cancer chemotherapy. In many cases, exposure of cells to toxic chemicals results in the concentration of these components after an initial depletion to become elevated either in a transient or

permanent manner. The resistance of these cells to further drug administration may be a result of these changes but perhaps more importantly may reflect an underlying protection mechanism (stress response) in eukaryotic cells. The potential of antagonizing this response or otherwise manipulating GSH and GSH-dependent enzymes in order to sensitize tumours to anticancer drugs represents an intriguing area for future research.

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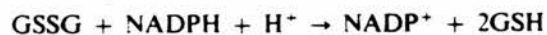
Glutathione reductase from *Escherichia coli*: mutation, cloning and sequence analysis of the gene

RICHARD N. PERHAM

Department of Biochemistry, University of Cambridge,
Tennis Court Road, Cambridge CB2 1 QW, U.K.

Glutathione reductase (EC 1.6.4.2) is a member of an important class of enzymes, the flavoprotein oxido-

reductases. Other members are thioredoxin reductase (EC 1.6.4.5), dihydrolipoamide dehydrogenase (EC 1.6.1.1). Glutathione reductase catalyses the reduction of oxidized glutathione by NADPH:



Reduced glutathione plays a critical part in the maintenance of reduced thiol groups in the cell and has particular import-

Abbreviation used: kbp, kilo base-pair.

(Eds) T.J. Mantle, C.B. Pickett and J.D. Hayes.

GLUTATHIONE S-TRANSFERASE
EXPRESSION IN NORMAL AND TUMOUR
CELLS RESISTANT TO CYTOTOXIC
DRUGS

(Taylor & Francis
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C. Roland Wolf¹, Alex D. Lewis¹,
James Carmichael¹, John Ansell¹, David
J. Adams¹, Ian J. Hickson², Adrian
Harris², F.R. Balkwill³, D.B. Griffin³ and John
D. Hayes⁴

¹Imperial Cancer Research Fund, Laboratory of Molecular
Pharmacology and Drug Metabolism, Department of
Biochemistry, Edinburgh

²Dept. Clinical Oncology, Newcastle General Hospital,
U.K.

³I.C.R.F. Interferon Laboratory, London

⁴Dept. Clinical Chemistry, University of Edinburgh

INTRODUCTION

The glutathione S-transferases (GST) play a central role in the protection of cells from cytotoxic and carcinogenic compounds either by catalyzing the conjugation of GSH with reactive electrophiles or by reducing reactive organic peroxides. The former reaction prevents electrophiles from reacting with macromolecules that possess critical cellular functions and the latter reaction protects against oxidative stress.

In cancer therapy there is increasing evidence that anticancer drugs interact with glutathione (GSH) in a manner which can either potentiate or decrease cytotoxicity (Arrick and Nathan, 1984, Wolf *et al.*, 1986). For example, there is evidence that cyclophosphamide and other nitrogen mustards form electrophilic derivatives that can form a thioether linkage with GSH (Berrigan *et al.*, 1982; Adams *et al.*, 1985). Alternatively, compounds such as adriamycin and bleomycin can be metabolised to toxic peroxides that may be reduced by GSH (Doroshow *et al.*, 1980).

The effectiveness of current cancer chemotherapy is determined by the relative toxicity of the drug to normal tissues, in particular the bone marrow and gut, relative to the toxicity to the tumour cells. Tumour cell susceptibility to these agents can be broadly split into the following three categories: a) tumours which can be

cured with anticancer drugs; b) tumours which are initially sensitive to therapy but after an initial response become resistant to the drugs, i.e. acquired resistance; c) tumours which are intrinsically resistant to therapy. In the case of acquired drug resistance there are several possible mechanisms which would explain the generation of drug resistant cells; two of these involve selection processes. The first mechanism requires the existence of a heterogeneous cell population within the tumour that exhibit differential sensitivities to cytotoxic drugs. In this instance initial treatment with an anticancer drug results in the destruction of sensitive cells leaving predominantly viable drug-resistant cells. The drug-resistant cells become the major cell population within the tumour. In the second proposed mechanism of resistance, administration of the drug may cause phenotypic changes either in enzymes or membrane structure within the tumour (possibly also by selection) that makes the cell resistant to further drug treatment. In the second case adaptive change accounts for the acquired drug resistance. In view of their role in detoxification we have been investigating the potential role of glutathione S-transferase (GST) in the acquisition of drug resistance in tumour and normal cells. The studies have mainly focused on the expression of the GST in cells of the bone marrow and tumour cells sensitive or resistant to chemotherapeutic agents.

2. METABOLISM AND RESISTANCE TO CYTOTOXIC DRUGS

The GST enzymes represent a supergene family of proteins. The three cytosolic classes of GST are dimeric and in man comprise binary combinations of α (26.0 kDa), γ (26.7 kDa) and π (24.8 kDa) type subunits respectively; (Hayes and Mantle, 1986) the three classes that comprise α , γ and π subunits have been classified as basic, neutral and acidic GST, respectively. Two basic α -type subunits exist and these have been designated B_1 and B_2 (Stockman et al., 1985). A separate microsomal GST has also been described and consists of three polypeptides, each of 17.0 kDa. The μ subunit possesses a unique substrate specificity and therefore their relative concentration will determine the cell's detoxification capacity. In spite of the known importance of these proteins in determining the

effectiveness of cancer chemotherapeutic agents there have been very few studies to establish the importance of GST-mediated detoxification and the relative role of individual isozymes.

In recent studies, we have obtained evidence that GST may play a role in the detoxification of mitoxantrone (Wolf et al., 1986), an anthraquinone derivative which is currently undergoing clinical trials for the treatment of breast cancer. In this case, evidence has been obtained indicating conjugation of mitoxantrone with GSH catalysed by microsomal GST and glucuronic acid, catalysed by UDP glucosyl transferase. These two reactions may represent important detoxification pathways for anthraquinones. The formation of the glutathione conjugate requires the presence of hepatic microsomes and NADPH and could be inhibited by carbon monoxide, indicating that a cytochrome P-450 oxidation was first required. The formation of the GSH conjugate was inhibited either by boiling the microsomes or by incubation with the GST substrates, hexachloro-1,3-butadiene and 1-chloro-2,4-dinitrobenzene (CDNB). The structure of the mitoxantrone-GSH conjugate is still unknown. Conjugate formation was not found to be increased by inducers of cytochrome P-450 or by the addition of hepatic cytosol to the incubations indicating that the reaction catalysed by microsomal GST, is the rate limiting step.

This finding may indicate that metabolites of the anthraquinones are specific substrates for the microsomal GST. Therefore, by analogy, other quinnone anticancer drugs, e.g. adriamycin, may also be detoxified by this mechanism. There is also some evidence which indicates a role for the GST enzyme system in the detoxification of cyclophosphamide metabolites (McGowan and Fox, 1986).

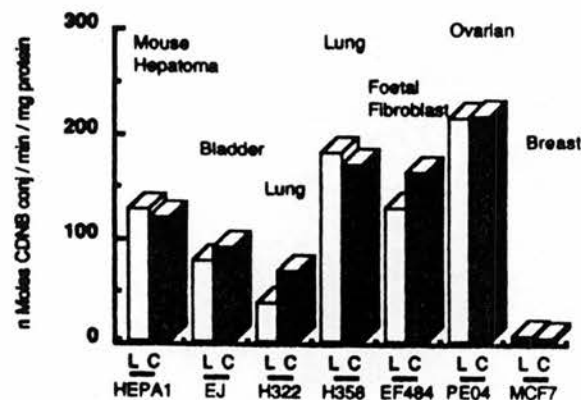
3. EXPRESSION OF GST IN TUMOUR CELLS

Relatively little is known about GSH levels and GST expression in tumour cells. A recent report by Kodate et al (1986) suggests that the acidic π GST subunit is highly elevated in human colonic adenomas and adenocarcinoma tissue. In the cell lines derived from different types of mammalian tumour we have found a large variation in GST activity determined using CDBN as substrate Figure 1.

* [The human cytosolic GST can be classified as basic (α - ϵ), neutral (μ , ψ) and acidic (ρ , λ , π). The autologous proteins in rat and mouse comprise polypeptides of similar apparent Mr i.e. Ya, Yb and Yf-type subunits but possess markedly different pI values. In this text the human cytosolic enzymes are referred to as basic, neutral and acidic GST whereas GST from other species are designated by the type of subunit they comprise]

Figure 1. Activity of various tumour cell lines in the conjugation of 1-chloro-2,4-dinitrobenzene with GSH

Confluent cells (C) or cells in logarithmic growth (L) were harvested and sonicated in 0.5ml, 50mM phosphate buffer, pH 7.4. The samples were centrifuged and the supernatant taken for GST assay using CDNB as substrate.

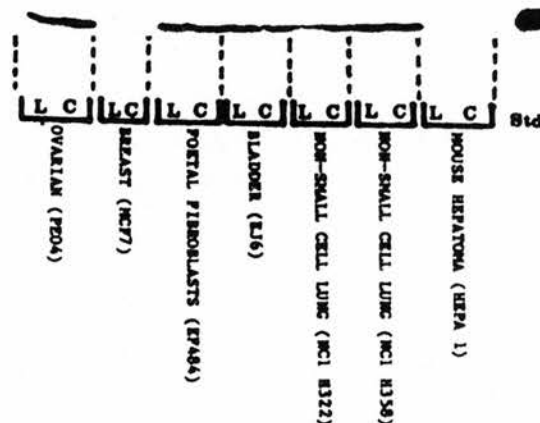


The CDNB-GSH-conjugating activity in the human breast cancer line MCF7 was extremely low and was approximately 100-fold less than was observed in either the human ovarian cell line PE04 or the human lung non-small cell carcinoma line H358. In these studies no significant difference was observed between CDNB activity in confluent cells versus cells in logarithmic growth. The very low level of activity

in the MCF7 cells is of interest in view of the reports in the increase in GST activity in these cells made resistant to adriamycin (Batist et al., 1985). Western blots of the soluble fraction from these cell lines using antibodies raised against the human basic (ϵ , YaYa) neutral (μ , YbYb) and acidic (λ , YfYf) GST showed high levels of the acidic YfYf-containing enzyme in most of the cell lines studied Figure 2.

Figure 2. Expression of acidic Glutathione transferase (Yf Yf) in various tumour cell lines

30 ug of soluble cellular protein was loaded per track and Western blots were carried out using a peroxidase labelled second antibody and 4-chloro-1-naphthol as substrate. All the cell lines examined are of human origin, with the exception of HEPA 1.



In agreement with the data obtained with CDNB the ovarian adenocarcinoma cell line PEO4 (Wolf et al., 1985) and the H358 cell lines had the highest levels of the acidic subunit. The human bladder carcinoma cell line (EJ) also contained high levels of the Yf polypeptide. The mouse hepatoma cell line contained extremely low amounts of the Yf subunit and no visible band could be seen in MCF7 cells.

None of the cell lines shown in Figure 2 contained any detectable Ya GST basic. However, preliminary evidence does indicate that the Ya-type subunits are expressed in certain CHO cells (see below). The neutral enzyme γ (Yb Yb), was only detected in the MCF7, the Hepa 1 and foetal fibroblast cell lines. However, as GST is polymorphic in man (Hayes et al., 1986; Seidegard et al., 1985) the failure to identify Yb subunits in PEO4, EJ6, NCI H322 and NCI H358 lines may simply reflect the fact that approximately 53% of the population fail to express this GST. Surprisingly, the GST- γ (Yb Yb)-IgG recognised a second polypeptide of faster mobility (approx. 23.5 kDa) in all the samples tested. Whether this represents another GST enzyme subunit remains to be determined.

These data show that different tumour cell lines show marked differences in their GST content. Indeed, we have initial information which indicates that this is also the case in solid tumours samples. These variations in the levels of particular GST enzymes could be a reason why certain tumour types are refractory to chemotherapy.

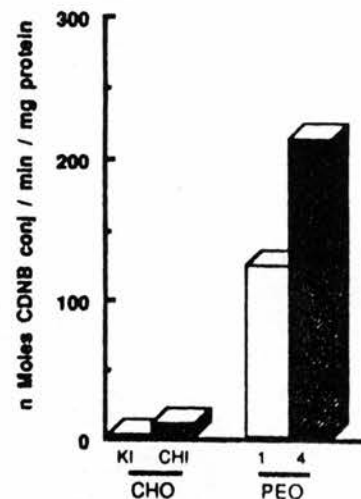
4. EXPRESSION OF GST IN ACQUIRED DRUG RESISTANCE

In addition to studies of the constitutive levels of GST in tumours, investigations into GST regulation in tumour cells which have become resistant to cytotoxic drugs have also been undertaken. In this regard two models have been established. Firstly two ovarian adenocarcinoma cell lines (PEO1 and PEO4), derived from peritoneal ascites samples, have been obtained from a patient, before and after she became resistant to chemotherapy (Wolf et al., 1985).

Karyotyping showed the two lines to be of the same clonal origin and *in vitro* clonogenic assays showed them to have different sensitivities to a variety of drugs including cis-platinum, adriamycin. The other model studied was a Chinese hamster ovary cell line that was selected *in vitro* for resistance to chlorambucil (Robson et al., 1986). Comparison of GSH concentrations and GST enzyme levels as well as the levels of a variety of other enzymes involved

in glutathione homeostasis showed significant differences between the resistant and sensitive cell lines.

Figure 3 Comparison of glutathione transferase activity in drug sensitive and drug resistant cell lines
Activity was measured using CDNB as substrate



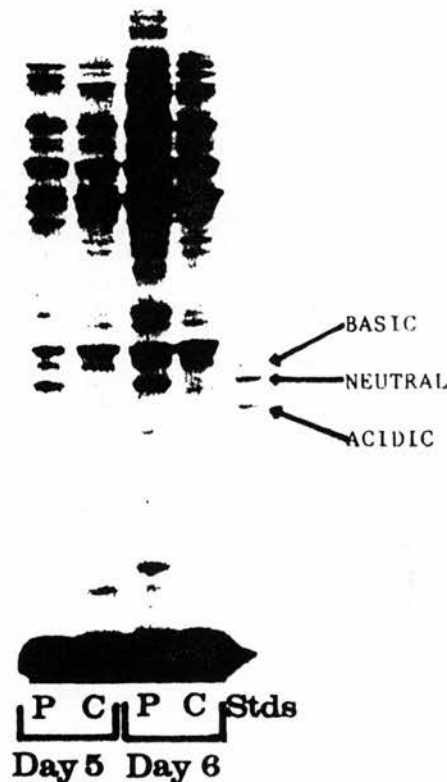
The drug sensitive ovarian cell line PEO1 contained approximately half the glutathione content, glutathione S-transferase and glutathione peroxidase activity compared to PEO4 (Figure 3). In addition γ -glutamyl transpeptidase was ten-fold higher in the drug resistant PEO4 cells. The drug-resistant CHO cells also had elevated glutathione and GST levels, the activity towards CDNB being seven-fold higher than in the chlorambucil sensitive cell line. Initial studies indicate that in both the ovarian and CHO cell lines the Yf GST subunit is elevated in the drug resistant cells. In addition, dot blot analysis indicated the Ya GST subunit is significantly elevated in the drug-resistant CHO lines. Whether the elevation in the levels of these isozymes is the primary reason for the drug-resistance of these cells is still unclear. However, it is interesting that in other cell models, where cells

exposed to cytotoxic compounds become resistant to further cytotoxic insult, for example, in other tumour cell models (Batist et al., 1985); Wang and Tew, 1985; Tew et al., this issue) or in preneoplastic nodules in the liver (see also below), glutathione and GST levels have also been shown to be markedly elevated (Kitahara et al., 1985; Buchmann et al., 1985). Although a great deal of emphasis has been placed on the acidic Yf GST subunit in this regard the changes observed are not restricted to this protein but are also observed in Ya and Yb type subunits (Buchmann et al., 1985). The changes in GST levels may therefore represent a stress response.

5. REGULATION OF GST IN NORMAL CELLS AS A STRESS RESPONSE

The response of normal cells to stress is not as easy to investigate as the changes observed are often transient. The stress response of normal cells towards cytotoxic drugs may be studied using cyclophosphamide-primed cells. In this model it has been observed that when mice are dosed with a low 'priming' dose of cyclophosphamide, they then become resistant to a subsequent potentially lethal dose given 5 to 6 days later (Miller et al., 1975). The lethal effects of cyclophosphamide have been associated with toxicity to the granulocytes of bone marrow. Therefore adaptive changes appear to have occurred in this cell population which have made them resistant to further cyclophosphamide treatment. Recent studies have shown that at the time points where maximum protection is obtained against cyclophosphamide significant elevations in granulocytic GSH and GST levels are observed (Adams et al., 1985; Carmichael et al., 1986). As both GSH and GST have been implicated in the detoxification of the cytotoxic metabolites of cyclophosphamide the changes in the levels of these components could represent the mechanism of resistance. SDS/PAGE analysis of bone marrow proteins following 'priming' with cyclophosphamide is shown in Figure 4. An elevation of a polypeptide that comigrated with authentic rat Yb subunits was observed. An elevation in the levels of the Yb subunit was confirmed by Western blot experiments (Fig 5). Slight elevation in the Ya subunit was also observed. The elevation in the Yb subunit was shown to occur within the granulocyte cell population (not shown) and could be observed in peripheral cells as well as cells from the bone marrow.

Figure 4 SDS-PAGE of mouse bone marrow in animals treated 5 or 6 days previously with cyclophosphamide (75mg/kg). 100 ug of marrow soluble protein was loaded per track. C=control, P=cyclophosphamide-primed. The standards are rat lung GST (Yf, Yb, Yc, Acidic, neutral and basic respectively)



The granulocytes fail to express the Yf subunit; which could explain why Yf subunits were not elevated in these cells. By contrast, lymphocytes contained high levels of the Yf subunit and low levels of the Yb subunit. However, the levels of GST in these cells were unchanged following cyclophosphamide priming. The changes observed in the marrow granulocytes were only observed over a two to three day period. Because of the limitations in sampling it, it was not possible to assess whether the increase levels of GST were transient within a cell, or whether the return to basal levels was because the affected granulocytes had been released into the circulation.

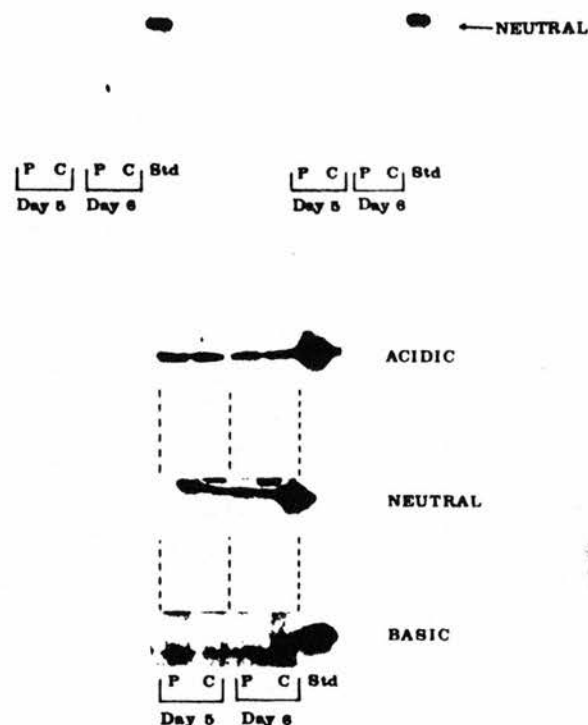
6. ENDOGENOUS REGULATION OF GST

Other models have been described where GST levels are elevated in normal cells following administration of cytotoxic compounds. For example, in the liver following the administration of hepatotoxin carbon tetrachloride (DiSimplicio, 1982). Such examples also indicate GST enzymes as stress response proteins. We are currently studying the regulation of specific GST subunits in the alternative models such as heat shock and oxidative stress. As a result of its potential role in drug resistance and preneoplasia there is a great deal of current research effort being directed towards the Yf GST subunit. The resistance of cells to viruses through the action of interferon could also be considered a stress response.

We initiated studies on the effects of interferon on GST expression not for this reason however, but primarily because of the potential use of interferons in combination with cytotoxic drugs in cancer therapy. In previous reports interferons have been shown to cause significant reduction in hepatic cytochrome P-450 levels (Renton et al., 1978, Balkwill et al., 1984) and effects on either cytochrome P-450 or GST levels could significantly affect the disposition and effectiveness of the drugs used. In a preliminary study it was found that interferons could affect GST activity (Balkwill et al., 1984). We have subsequently quantitated the levels of various hepatic glutathione transferases in mice (Adams et al., 1987) following administration of alpha/beta interferon. Interestingly, in a manner similar to the cytochrome P-450's, the levels of the various GST enzymes were reduced. In particular proteins which reacted with the human basic (B₁ or Ya-type subunits and the rat Yb₂ subunits. The

Figure 5. Western blots of mouse bone marrow following drug priming

The neutral Yb antibody was used for the top blots. The bottom panel shows three blots that were probed using antibodies indicated and visualized using I¹²⁵-labelled protein A followed by autoradiography
C=control P=cyclophosphamide primed



most significant change, however, was in the levels of the Yf subunit which was elevated five to seven fold following interferon administration. This was paralleled by an increase in the ethacrynic acid - GSH-conjugating activity; ethacrynic acid is a substrate for the Yf subunit. The increased levels of the Yf subunit may not be part of the type of stress response described above, but the increase is of interest because conventional exogenous inducing agents do not affect the hepatic levels of the Yf-containing enzyme. The GST Yf subunit may have additional functions in cells other than the conjugation of electrophilic foreign compounds.

In conclusion, glutathione, glutathione transferases and various other glutathione-dependent enzymes have been shown to be elevated in a variety of cells exposed to cytotoxic chemicals. These changes are observed in a transient manner in a variety of normal cells and in an apparently immortalized fashion in preneoplasia and drug resistant tumour cells. (see, Adams et al., 1985; Carmichael et al., 1986). The apparently universal nature of this response suggests that the elevated levels of this group of enzymes represent a type of stress response. In view of the importance of these components in the protection of cells from environmental chemicals, or, in the case of tumours, from anticancer drugs, further characterization of these changes, the underlying mechanisms which regulate them and how they relate to cellular resistance represent important areas for further study.

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GLUTATHIONE-S-TRANSFERASES IN ALKYLATING AGENT RESISTANT CELLS

Margie L. Clapper, Amy L. Buller,
 Thomas M. Smith and Kenneth D. Tew

Department of Pharmacology
 Fox Chase Cancer Center
 7701 Burholme Avenue
 Philadelphia, PA 19111

The diverse response of a heterogeneous tumor cell population to chemotherapy has continued to complicate effective anticancer treatment. Selection of subpopulations with increased homogeneity has resulted in both the establishment of resistant cell populations following drug exposure (acquired resistance) and the existence of tissue types refractory to chemotherapy (intrinsic resistance). Several alterations in cell function have been proposed as explanations for cellular resistance and these include gene amplification, decreased drug uptake, increased DNA repair, decreased drug metabolism and increased drug detoxification.

The role of detoxification enzymes in cellular resistance has recently received much consideration because of numerous reports of elevated glutathione-S-transferase (GST) activity in drug resistant tumor cells. For example, the multidrug resistant MCF-7 breast carcinoma cell line exhibits a 45 fold increase in GST activity when compared to the parent cell line (Batist 1986); Walker 256 rat breast carcinoma cells resistant to nitrogen mustards demonstrate elevated levels of GST as compared to sensitive cells of the same cell line (Wang and Tew 1985). According to Carmichael et al. (1985), administration of a low dose of cyclophosphamide results in increased GST activity in mouse bone marrow granulocytes which may contribute to observed protection against a subsequent lethal cyclophosphamide dosage. Whether this response to drug treatment is specific to GST or part of a more generalized cell response to stress remains to be determined. However, examination of liver GST demonstrated specificity in the enhanced expression of particular GST



CELLULAR HETEROGENEITY AND DRUG RESISTANCE IN TWO OVARIAN ADENOCARCINOMA CELL LINES DERIVED FROM A SINGLE PATIENT

C. Roland WOLF^{1,4}, Ian P. HAYWARD¹, Sandra S. LAWRIE¹, Karin BUCKTON², Margaret A. MCINTYRE³, David J. ADAMS^{1,5}, Alexander D. LEWIS^{1,4}, Angela R.R. SCOTT^{1,4} and John F. SMYTH^{1,6}

¹Imperial Cancer Research Fund, Medical Oncology Unit; ²Medical Research Council, Clinical and Population Cytogenetics Unit; and ³Department of Pathology, Western General Hospital, Edinburgh, UK.

Two ovarian cell lines were derived from the ascites of a patient before and after the onset of resistance to chemotherapy involving cis-platinum, chlorambucil and 5-fluorouracil. Characterization of these lines shows them to have various features in common and some significant differences. Cytologically the lines cannot be distinguished and they both contain high concentrations of oestrogen receptor. However, they do differ with respect to their growth characteristics, karyotype, glutathione content and sensitivity to cis-platinum. The karyotypes of the 2 lines show several marker chromosomes in common but the resistant line contained a chromosome 8 and a 17 which were absent from the earlier sensitive line. This suggests a clonal origin with subsequent divergence to a heterogeneous population.

Ovarian adenocarcinoma is responsive to a variety of chemotherapeutic agents (Rodenberg and Cleton, 1984). However, the remissions which result from treatment are often short, and following relapses the patients are usually resistant to subsequent chemotherapy (Rodenberg and Cleton, 1984). There are numerous possible explanations for the refractoriness of tumours to treatment (Hill, 1982). Firstly, there may be alterations in drug disposition not related to the tumour cells themselves. Secondly, there is the possibility that the tumour cells, when exposed to cytotoxic compounds, acquire resistance to them. The phenomenon of acquired resistance has been extensively studied and is well characterized for many cell culture model systems (Schimke, 1984; Louie *et al.*, 1985). In the majority of reports, resistant cells have been produced *in vitro* by selection, *i.e.* by growing the cell lines in the presence of the drug under investigation (Fojo *et al.*, 1985). In view of the relative difficulty in obtaining tissue samples and transferring them successfully into culture (Bertonecello *et al.*, 1982), only a few reports have unequivocally indicated that acquired drug resistance is an important factor in the failure of chemotherapy (Bell *et al.*, 1985). A third explanation for resistance is the presence of minor cell populations within the original tumour which are intrinsically more resistant to chemotherapy and which, following initial treatment, become the major population within the tumour. This represents the basis of the Goldie and Coldman model (1979) and is supported by many reports of tumour-cell heterogeneity (Heppner, 1984).

In order to investigate these possibilities, we have obtained ascites samples from a patient before and after the onset of clinical drug resistance. Two cell lines have been established from these samples, characterized, and tested for drug sensitivity.

MATERIAL AND METHODS

Source of ascites samples and cell lines

The patient, who was diagnosed as having stage-III ovarian adenocarcinoma in April 1980, was treated with 7 courses of 5-fluorouracil (500 mg/m² i.v.), cis-platinum (30 mg/m² i.v.) and chlorambucil (0.1 mg/kg oral) over a period of 7 months. A complete response—determined by second-look laparotomy—was obtained and no further treatment was given until

relapse 14 months later. A sample of ascitic fluid was obtained at this time and the cells were isolated and placed into tissue culture (cell line PE01). The patient then received 6 further courses of treatment (February-August 1982) as described above and responded well with clearing of ascites. A computerized axial tomography scan of the abdomen performed in August 1982 was normal. In November 1982 the patient relapsed with further ascites and she was then treated with a higher dose of cis-platinum (100 mg/m²) but did not respond. A cell line was derived from a second ascites sample (PE04) taken in December 1982. The patient died in February 1983.

The cell lines were derived in the following manner. The freshly obtained ascites samples were mixed with 100 units of preservative-free heparin and the cells sedimented by centrifugation. After the cells had been washed twice with phosphate-buffered saline (PBS), red cells were removed using a Ficoll Hypaque gradient. Tumour cells at the interface were aspirated off, washed twice with PBS and checked for viability by nigrosin staining. Aliquots of 10⁵ cells per ml were then cultured at 37°C, 100% humidity and 5% CO₂ in RPMI 1640 + 10% foetal calf serum (v/v) with added insulin (2.5 µg/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml) and 3-[N-morpholino]propanesulphonic acid (12.5 mM). PE01 and PE04 were subcultured after 21 and 4 weeks respectively. Confluent cultures were passaged by 1:5 or 1:10 splits. Periodic assays for Mycoplasma were negative.

Cytology

Cells at passages 1 and 79 of PE01 and 2 and 48 of PE04 were harvested and grown on sterile 22 × 22 mm coverslips in 35-mm wells (6-well plates, Sterilin, Teddington, UK). When regular and satisfactory growth was obtained, the cells were fixed and stained with PAP to reveal gross morphology or with PAS-diacetate to stain for mucin.

Karyotyping

Chromosome preparations were made from the patient's peripheral blood as well as from cell lines PE01 and PE04 at

⁴Present address: Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, Dept. of Biochemistry, Hugh Robson Building, George Square, Edinburgh.

⁵Present address: Dept. of Microbiology, University of Leeds, Leeds, UK.

⁶To whom reprint requests should be sent.

Abbreviations: HSR, homogeneously staining region; PBS, Dulbecco's phosphate-buffered saline; PAP, Papanicolaou; PAS-diacetate, periodic acid Schiff.

various passages in culture. A minimum of 20 spreads of each preparation were examined by staining with spermidine bis-acridine by the Q-band technique of Van de Sande *et al.* (1979).

Drug sensitivity assay

Drug sensitivity assays were carried out at the same time for the 2 cell lines. Cells were taken at passages 77 to 82 and 46 to 51 for PE01 and PE04 respectively.

(1) Clonogenic assay in soft agar

The *in vitro* soft-agar assay of Courtenay *et al.* (1978) was used. A single-cell preparation was obtained by vigorous aspiration and passage through a 19-gauge needle. Viability was checked with nigrosin stain, then 5×10^3 and 1×10^3 cells/ml in a test tube for PE01 and PE04 respectively were incubated with drug in the agar for 21 days, with 1 ml of fresh medium added on days 7 and 14. Colonies of more than 50 cells were scored visually.

(2) Clonogenic assay on plastic

Assay conditions were modified from those described by Parsons and Brown (1979) to suit these ovarian cell lines. Cells were plated out in 35-mm wells (6-well Linbro plates, Flow, Irvine, Scotland) at 2×10^3 and 10^3 cells/well for PE01 and PE04 respectively in medium containing 1 mM pyruvate to give 100 to 200 colonies in control wells. After 2 days, when cells had firmly attached but had not yet divided, drugs were added to wells in triplicate and left on for 3 days. Medium was changed every 2 to 3 days. After 12 to 14 days colonies were counted visually.

Colony-forming efficiency and doubling time

The colony-forming efficiency of the lines in agar and on plastic was determined in the Courtenay assay and the assay on plastic as described. Doubling time was determined by plating at 5×10^3 cells per well in a 24-well plate (NUNC, Gibco, Paisley, UK) and harvesting rows of 4 wells at the same time each day for 12 days. Harvested cells were counted with a Coulter counter.

Glutathione and glutathione transferase assays

Glutathione levels were determined by HPLC following derivitization with monobromobimane (Gaetjens *et al.*, 1984). Glutathione transferase activity with 1-chloro-2,4-dinitrobenzene as substrate was determined by the method of Habig *et*

al. (1974). Cells used were in log phase and the values were expressed per 10^6 viable cells.

Hormone receptor assays

Oestrogen and progesterone receptor assays were carried out on confluent cell cultures maintained for 6 to 10 days in steroid-stripped medium (Hawkins *et al.*, 1981; Hamilton *et al.*, 1983).

DNA content

DNA content was measured on a fluorescence-activated sorter using human lymphocytes as an internal diploid marker. Only a single G₁ peak was observed.

RESULTS

(1) Cytology

The cytology of the ascites samples and of the cell lines derived from them is shown in Figure 1. In contrast to the original ascites sample taken in April 1980, the 2 later samples from which cell lines PE01 and PE04 were derived were largely devoid of both macrophages and lymphocytes. The ascitic fluids contained groups of cells as well as single cells of adenocarcinomatous type, characterized by large nuclei, eccentrically situated in the cytoplasm and occasional signet-ring cells due to the presence of a large vacuole containing mucopolysaccharide. The latter property is consistent with ovarian metastases. Cytological examination of the cell lines showed them to be very similar to the cells of the ascitic fluids (Fig. 1d and e). Both cell lines had large oval nuclei with prominent, sometimes multiple, nucleoli; they contained mucin-secreting cells and were mucin-secreting. No cytological differences between the cell lines or between different passages of the cell lines was apparent even at the ultrastructural level by electron microscopy (results not shown).

(2) Drug sensitivity

The sensitivity of PE01 and PE04 to the cytotoxic drugs used clinically, *i.e.* cis-platinum, chlorambucil and 5-fluorouracil, tested using clonogenic assays in agar and on plastic, is shown in Figures 2-4. Two different assays were used to give us greater confidence in the significance of the drug-sensitivity differences between the 2 cell lines. A 3-fold difference in sensitivities of the lines to cis-platinum was measured using either assay. Both assays gave similar results, the LD₅₀ values

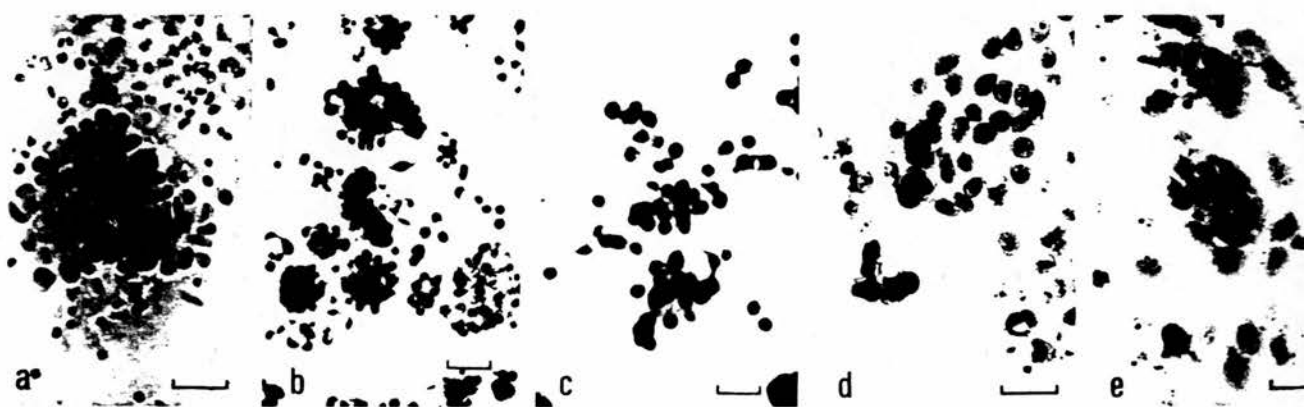


FIGURE 1 - Cytology of ascites and cell line samples. Samples were prepared and stained with Papanicolaou stain as described in "Materials and Methods." (a) Ascites cells, April 1980. (b) Ascites cells, February 1982. (c) Ascites cells, December 1982. (d) Cell line PE01, passage 79. (e) Cell line PE04, passage 48. (Scale bar represents 40 μ m).

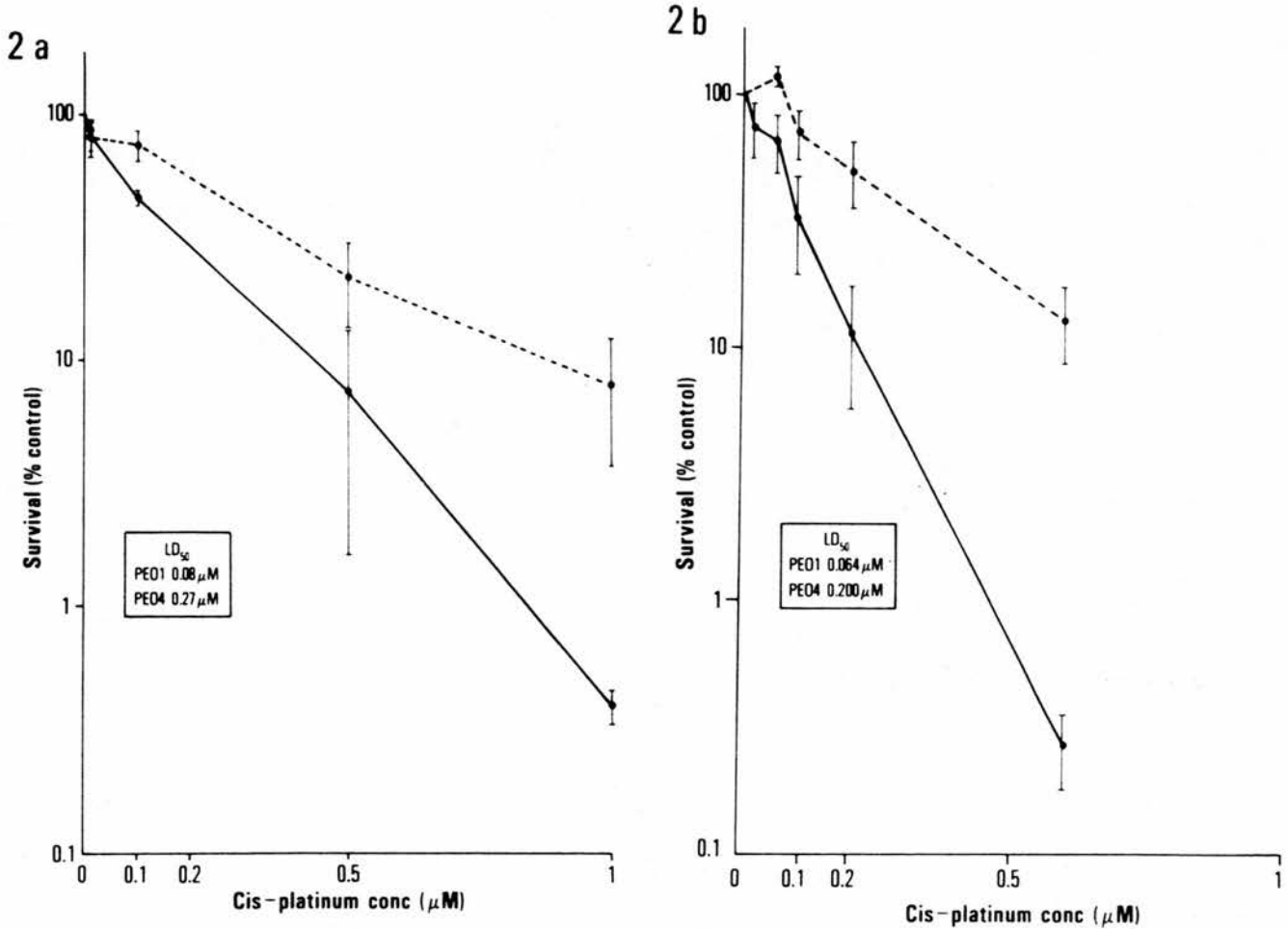


FIGURE 2 - Drug sensitivity for cell lines PE01 and PE04 using cis-platinum. (a) Clonogenic assay in soft agar according to Courtenay *et al.* (1978). Points, means of 1-2 experiments PE01 and 2-6 experiments PE04; bars represent the standard error. (b) Clonogenic assay on plastic. Assay conditions as described in "Material and Methods" section. Points, means of 2-4 experiments; bars represent the standard error.

being $8.0 \times 10^{-8}M$ and $2.7 \times 10^{-7}M$ in the agar assay and $6.4 \times 10^{-8}M$ and $2.0 \times 10^{-7}M$ in the assay on plastic for PE01 and PE04 respectively. For chlorambucil the sensitivity difference was 3-fold in the plastic assay and only 1.3-fold in the assay on agar. In the case of 5-fluorouracil, no differences between the cell lines were measured by either assay. Although there was more variation between experiments in measuring sensitivity to 5-fluorouracil, probably due to variation in the nucleosides in serum batches, no differences between the cell lines within an experiment were observed. Both cell lines appeared more sensitive to 5-fluorouracil in the agar assay.

(3) Cell line characteristics

A summary of the general properties of the 2 cell lines is shown in Table I and Figure 5. PE04 had a higher colony efficiency. Both glutathione and glutathione transferase activity, components central to the deactivation of alkylating agents (Mitchell *et al.*, 1982), were approximately twice as high in PE04 as in PE01. Both cell lines had high levels of oestrogen receptor, the value for PE04 being slightly higher than that for PE01, and undetectable levels of progesterone receptors after 10 days' culture in steroid-stripped medium (Hamilton *et al.*, 1983).

(4) Karyotype

Karyotypes of the cell lines and peripheral blood are shown in Figure 6.

The cells from the peripheral blood showed a normal 46,XX karyotype even after chemotherapy, whereas the cell lines from the ascites were hypodiploid. However, their DNA content was slightly greater than that of normal diploid human lymphocytes. The modal chromosome numbers from different spreads varied between 39 and 42. Examples of the chromosome complement of PE01 and PE04 with 41 chromosomes are illustrated (Figs. 6a,b). Figure 6a shows a Q-banded karyotype made from a normal peripheral blood cell taken from the patient 4 weeks before she died. The cultured ascites cells, in addition to being hypodiploid, contained many abnormal chromosomes. The chromosomes that are considered to be normal are marked with a white square. PE01 has only 20 such chromosomes. None of the cells analysed contained a normal 8 or 17 chromosome or any material that could be considered as coming from either of these chromosomes, although this cannot be excluded because of many rearranged chromosomes. PE01 had 12 consistent abnormalities which were: both 3s inv(3)(p-q+), 5p-, 9p+, 11p+, both 13s 13q- and 13q+, 22q+ and 4 chromosomes of unknown

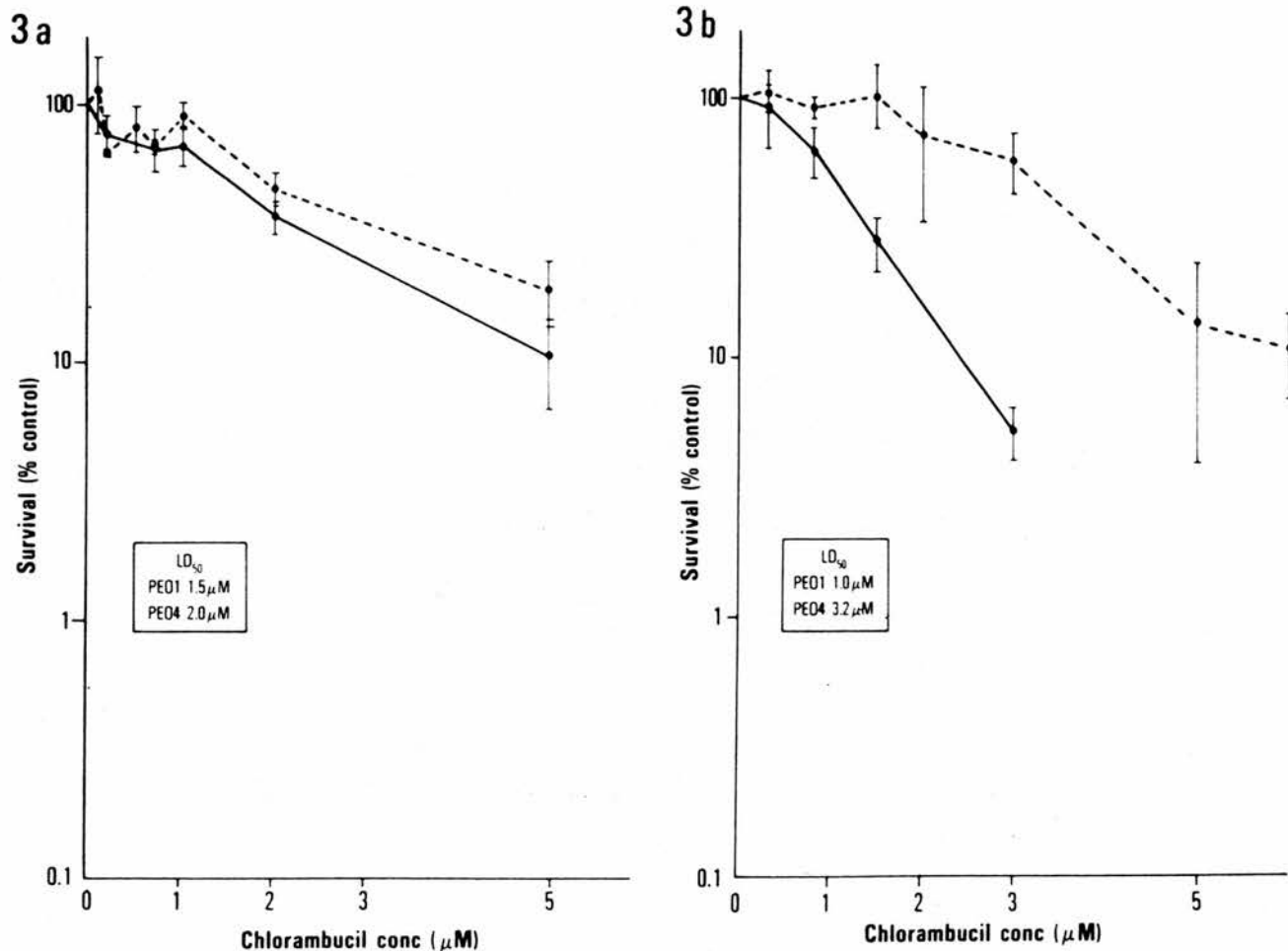


FIGURE 3 - Drug sensitivity for cell lines PE01 and PE04 using chlorambucil. Legend as for Figure 2. Points, means of 2-5 experiments; bars represent the standard error.

origin. The number and appearance of the other abnormal chromosomes varied. Of the 4 chromosomes of unknown origin, 2 marker chromosomes had a similar appearance—2 prominent bands on the distal long arm but the pericentric chromatin which was very pale, uniformly staining and reminiscent of homogeneously staining regions (HSR). These regions were not C-band-positive. The 2 other chromosomes appeared to be isochromosomes, being metacentric with a very similar banding pattern on each side of the centromere, one being half the size of the other.

In contrast, PE04 had an apparently normal member of each chromosome pair, including a chromosome 8 and 17 but excepting an X and a 13 chromosome. However, the 13q-chromosome now has material translocated to the brilliant p11 which could make this chromosome complete although rearranged. Of the consistent abnormalities seen in PE01, the following are seen in PE04: *inv*(3)(p-q+) with the brilliant Q-band, 5p-, 9p+, 11p+, 13q+, 22q+, one of the marker chromosomes with the possible HSR; in many cells the smaller of the 2 isochromosomes was seen. PE04 had acquired a ring chromosome which was approximately the size of a D-group chromosome, and also other rearranged chromosomes.

The "normal" 22 in both PE01 and PE04 has lost the intense satellites. Deletion of satellites or the whole of an acrocentric

short arm is often found as a constitutional "abnormality" in normal individuals.

DISCUSSION

Two ovarian adenocarcinoma cell lines have been established from ascites obtained before and after the onset of resistance to chemotherapy. A marked difference was observed in the sensitivity of these lines to cis-platinum, one of the chemotherapeutic agents used for treating the patient. Although the difference in sensitivity of these lines to cis-platinum was only 3-fold, we believe that changes in drug sensitivity of this order of magnitude would be clinically important (Ozols *et al.*, 1984; Hamilton *et al.*, 1984). No change in drug sensitivity with *in vitro* passaging was observed. While not the only possible explanation, our *in vitro* data suggest that the tumour-cell population or subpopulations changed in the patient during the drug treatment and the difference in drug sensitivity could explain the resistance of this patient to further treatment. In support of such *in vivo* change is the fact that the PE04 cell line was established more rapidly and with much less difficulty than the PE01 line. The LD₅₀ levels reported here are at the lower end of the range for cis-platinum toxicity values (0.03-3 μM) reported in the literature for ovarian (Holzel *et al.*, 1985; Buick *et al.*, 1985; Van Putten *et al.*, 1986).

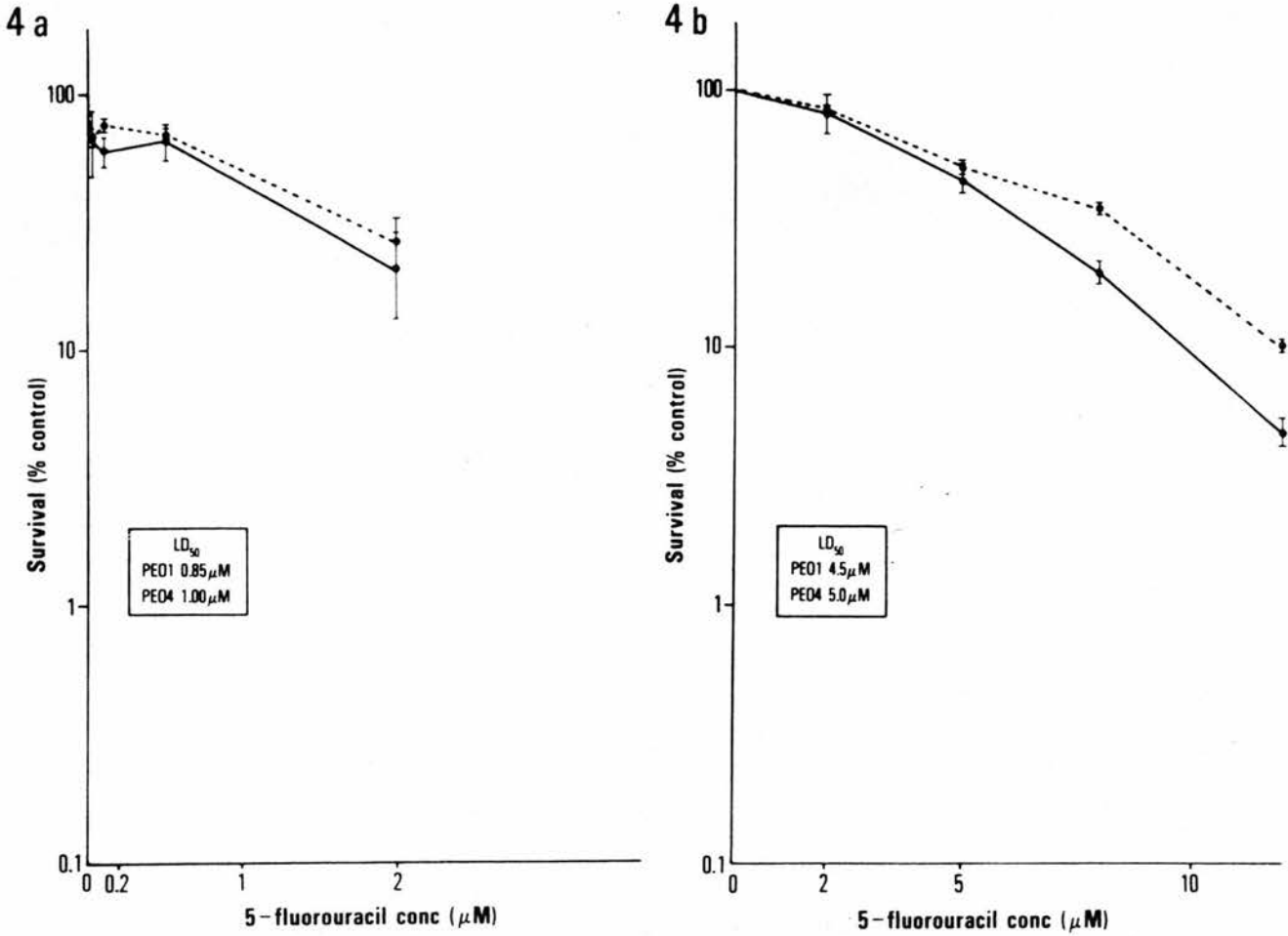


FIGURE 4 - Drug sensitivity for cell lines PE01 and PE04 using 5-fluorouracil. Legend as for Figure 2. A typical experiment is shown. Points, means of 5 replicates (agar) or triplicates (plastic); bars represent the standard error.

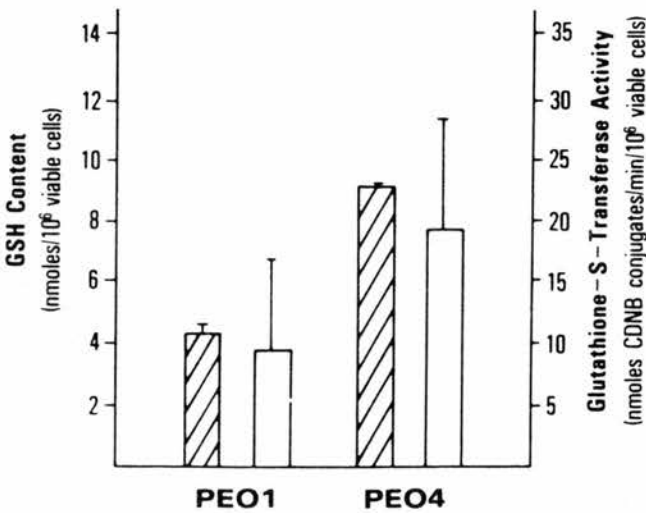


FIGURE 5 - Glutathione and glutathione-S-transferase activity in cell lines PE01 and PE04. Glutathione and glutathione-S-transferase activity was measured as described in "Material and Methods." Hatched columns represent glutathione content and open columns glutathione-S-transferase activity. Values are means of 3 separate experiments carried out in triplicate; bars represent the standard error.

TABLE I - CELL LINE CHARACTERISTICS

Characteristics	PE01	PE04
Doubling time (hr)	53	59
Mucin production	+	+
Colony-forming efficiency (plastic)	3.9 ± 2.3	8.1 ± 3.8
Colony-forming efficiency (agar)	3.9 ± 2.5	18.3 ± 10.4
DNA content	1.1 × diploid	1.1 × diploid
Modal chromosome number	41	41
Abnormal chromosomes	+	+
Oestrogen receptor (fmol/mg protein)	145 ± 34	203 ± 23
Progesterone receptor (fmol/mg protein)	Not detectable	Not detectable

and other human cell lines (B.T. Hill *et al.*, 1984) and below that reported for normal human bone-marrow cells (0.9 μM) (Umbach *et al.*, 1985).

Although the cell lines were cytologically similar, there were significant differences in their karyotypes. Chromosome abnormalities found in the tumour cells of other patients with ovarian carcinoma have also been bizarre, and inevitably some

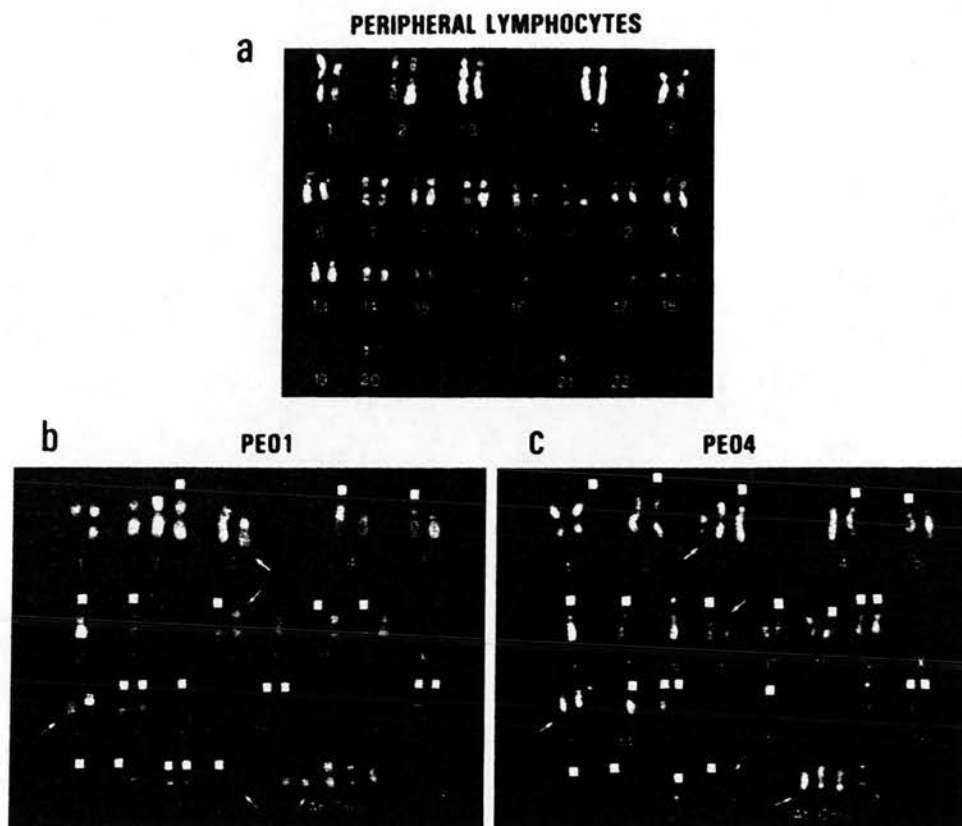


FIGURE 6 - Karyotypes of peripheral lymphocytes, and cell lines PE01 and PE04. (a) Q-banded karyotype of a normal cell from a peripheral blood culture, showing the Q-band polymorphisms. (b) Karyotype of a cell from PE01. The chromosomes marked with a white square are considered to be normal. The abnormal chromosomes which are arrowed are also found in PE04. (c) Karyotype of a cell from PE04. Symbols are as in (b) above. Chromosomes found only in PE04 are marked with a white spot for normal chromosomes and a cross for abnormal chromosomes.

of the chromosomal rearrangements in these tumours have involved the same chromosomes as in the present case (Hamilton *et al.*, 1983; Freedman *et al.*, 1978; S.M. Hill *et al.*, 1984; Kakati *et al.*, 1975; Van der Reit-Fox *et al.*, 1979; Woods *et al.*, 1979). However, we did not find the (6;14) translocation which, it is suggested, may characterize this carcinoma (Trent and Salmon, 1981), although we did observe a 3p-marker similar to that observed by others (Panani and Ferti-Passantonopoulou, 1985; Mackillop *et al.*, 1983).

No double minutes were found in these cells. The appearance of 2 marker chromosomes in PE01 was consistent with their carrying homogeneously staining regions (HSRs). HSRs have been, but need not necessarily be, associated with the acquisition of drug resistance (Schimke, 1984). It is interesting that the number of HSRs was reduced in PE04. PE04 appears to contain more "normal" chromosomes than the earlier PE01 culture. Since both cell lines have several rearranged chromosomes in common and were cytogenetically very heterogeneous, it could be argued that the 2 lines probably arose from a common ancestor, both diverged and co-existed in the body, but that PE01 was more susceptible to the initial drug treatment and was overgrown by PE04. It is very unlikely that PE04 evolved from PE01 as it would be almost impossible to regain a normal 8 and 17 having once lost them. The 2 cell lines therefore represent a model for the study of human tumour-cell heterogeneity. There is no evidence that the karyotype or properties of the cells changed during passaging *in vitro*.

Variation in sensitivity to cis-platinum due to tumour-cell heterogeneity has been observed in clonal human glioma line isolated from a single untreated tumour (Yung *et al.*, 1982). This sort of heterogeneity has been observed by others (Tsuru and Fidler, 1981) and has also been found in primary ovarian cancer samples (Siračsky, 1979). Our results, which suggest PE04 is not a lineal descendent of PE01, emphasize the importance and relevance of sub-population heterogeneity in drug resistance.

PE01 and PE04 contained extremely high levels of oestrogen receptor but undetected levels of progesterone receptors. Whether progesterone receptors could be induced by oestrogen, specifically excluded from the assay here, is as yet unknown. Oestrogen and progesterone receptor-positive ovarian tumours and cell lines have been reported (Hamilton *et al.*, 1983; Lazo *et al.*, 1984) but the levels of oestrogen receptor detected here are very high compared with those in most reports [e.g., mean of 18 and maximum of 163 fmol receptor/mg cytosolic protein in 56 ovarian tumour samples reported by Lazo *et al.* (1984)].

Two assays were used in this investigation of drug sensitivity, to increase our confidence in sensitivity differences measured. These were a relatively simple assay involving colony counting of attached colonies in 6-well plates, and the more complex assay involving colony growth in soft agar. The comparative sensitivity of the cell lines determined with these assays was similar although some differences were apparent. The reason why chlorambucil gave a larger sensitivity differ-

ence between PE01 and PE04 in the assay on plastic is unclear, but preliminary experiments suggest it may be related to the increased oxygen tension (20% O₂ vs. 5% O₂). The growth conditions in agar are clearly different from those in monolayer on plastic. In particular, anchorage dependence (Stephens *et al.*, 1980), oxygen tension (Gupta and Krishnan, 1982) and the addition of rat red blood cells in the Courtenay assay (Endresen *et al.*, 1985) have all been shown to affect chemosensitivity measurements for some cells. Moreover, the metabolic state of the cells when treated in suspension after trypsinization or after attachment is important (Twentyman, 1979).

These conditions may also explain the difference in sensitivity to 5-fluorouracil between the 2 assays, although other factors such as the presence of thymidine (Engelbrecht *et al.*, 1984) in the Ham F-12 medium (and not in RPMI-1640) used in the Courtenay assay or the length of time (3 days vs. 21 days) of drug exposure (Sobrero and Bertino, 1983) may also influence the results. Our data suggest that great care should be taken in relating *in vitro* assay results back to a patient since different assays can give different results, as here with chlorambucil. Both cell lines have been successfully xenografted in immune-deprived mice, and in experiments currently in progress comparisons are being made between *in vivo* and *in vitro* growth characteristics and drug sensitivities.

It is interesting to note the difference in glutathione levels in our cell lines. The role of glutathione in the protection of cells from cis-platinum is not clear. However, the role of this peptide in the detoxification of alkylating agents in general is well established (Mitchell *et al.*, 1982). Furthermore, some evidence suggests that thiol-containing compounds can in-

crease the survivals of cis-platinum-treated mice and also reduce both the kidney and gastrointestinal tract toxicity of cis-platinum (Allan *et al.*, 1986; Borch and Pleasants, 1979). The potential importance of intracellular thiols is illustrated in a recent report by Green *et al.* (1984) where an ovarian cell line resistant to melphalan was made sensitive to this compound by suppression of glutathione biosynthesis. In our cell lines the glutathione levels are similar to those of other ovarian cell lines as reported by Louie *et al.* (1985). Other workers have suggested that glutathione metabolism may be more important for melphalan and chlorambucil toxicity than for cis-platinum (Andrews *et al.*, 1985). However, the higher thiol level in PE04 may be a contributing factor to its increased resistance demonstrated in the present study.

The overall sensitivity or relative resistance of ovarian cancer to cis-platinum is multifactorial. Differences in thiol levels are just one of these factors. Tumour heterogeneity, demonstrated here cytogenetically, is also clearly important. The cell lines described in this report have been cryopreserved and satisfactorily retrieved from storage; they are now stable cell lines available for continued study. In view of the relative restrictedness of working with fresh ascites, we believe that these cell lines constitute an appropriate and useful model for the further study of mechanisms of drug resistance in this important human tumour.

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